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(54) Title: STREPTOCOCCUS PNEUMONIAE PROTEINS AND NUCLEIC ACID MOLECULES

(57) Abstract

Novel protein antigens from *Streptococcus pneumoniae* are disclosed, together with nucleic acid sequences encoding them. Their use in vaccines and in screening methods is also described.

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STREPTOCOCCUS PNEUMONIAE PROTEINS AND NUCLEIC ACID MOLECULES

The present invention relates to proteins derived from *Streptococcus pneumoniae*, nucleic acid molecules encoding such proteins, the use of the nucleic acid and/or proteins as antigens/immunogens and in detection/diagnosis, as well as methods for screening the proteins/nucleic acid sequences as potential anti-microbial targets.

Streptococcus pneumoniae, commonly referred to as the pneumococcus, is an important pathogenic organism. The continuing significance of *Streptoccocus pneumoniae* infections in relation to human disease in developing and developed countries has been authoritatively reviewed (Fiber, G.R., *Science*, **265**: 1385-1387 (1994)). That indicates that on a global scale this organism is believed to be the most common bacterial cause of acute respiratory infections, and is estimated to result in 1 million childhood deaths each year, mostly in developing countries (Stansfield, S.K., *Pediatr. Infect. Dis.*, **6**: 622 (1987)). In the USA it has been suggested (Breiman *et al*, *Arch. Intern. Med.*, **150**: 1401 (1990)) that the pneumococcus is still the most common cause of bacterial pneumonia, and that disease rates are particularly high in young children, in the elderly, and in patients with predisposing conditions such as asplenia, heart, lung and kidney disease, diabetes, alcoholism, or with immunosupressive disorders, especially AIDS. These groups are at higher risk of pneumococcal septicaemia and hence meningitis and therefore have a greater risk of dying from pneumococcal infection. The pneumococcus is also the leading cause of otitis media and sinusitis, which remain prevalent infections in children in developed countries, and which incur substantial costs.

The need for effective preventative strategies against pneumococcal infection is highlighted by the recent emergence of penicillin-resistant pneumococci. It has been reported that 6.6% of pneumoccal isolates in 13 US hospitals in 12 states were found

to be resistant to penicillin and some isolates were also resistant to other antibiotics including third generation cyclosporins (Schappert, S.M., *Vital and Health Statistics of the Centres for Disease Control/National Centre for Health Statistics*, **214**:1 (1992)). The rates of penicillin resistance can be higher (up to 20%) in some hospitals (Breiman *et al*, J. Am. Med. Assoc., **271**: 1831 (1994)). Since the development of penicillin resistance among pneumococci is both recent and sudden, coming after decades during which penicillin remained an effective treatment, these findings are regarded as alarming.

For the reasons given above, there are therefore compelling grounds for considering improvements in the means of preventing, controlling, diagnosing or treating pneumococcal diseases.

Various approaches have been taken in order to provide vaccines for the prevention of pneumococcal infections. Difficulties arise for instance in view of the variety of serotypes (at least 90) based on the structure of the polysaccharide capsule surrounding the organism. Vaccines against individual serotypes are not effective against other serotypes and this means that vaccines must include polysaccharide antigens from a whole range of serotypes in order to be effective in a majority of cases. An additional problem arises because it ahs been found that the capsular polysaccharides (each of which determines the serotype and is the major protective antigen) when purified and used as a vaccine do not reliably induce protective antibody responses in children under two years of age, the age group which suffers the highest incidence of invasive pneumococcal infection and meningitis.

A modification of the approach using capsule antigens relies on conjugating the polysaccharide to a protein in order to derive an enhanced immune response, particularly by giving the response T-cell dependent character. This approach has

been used in the development of a vaccine against *Haemophilus influenzae*. There are issues of cost concerning both the multi-polysaccharide vaccines and those based on conjugates.

A third approach is to look for other antigenic components which offer the potential to be vaccine candidates. In the present application we provide a group of proteins antigens which are secreted/exported proteins.

Thus, in a first aspect the present invention provides a *Streptococcus pneumoniae* protein or polypeptide having a sequence selected from those shown in table 2 herein.

A protein or polypeptide of the present invention may be provided in substantially pure form. For example, it may be provided in a form which is substantially free of other proteins.

In a preferred embodiment, a protein or polypeptide having an amino acid sequence as shown in Table 3 is provided.

The invention encompasses any protein coded for by a nucleic acid sequence as shown in Table 1 herein.

As discussed herein, the proteins and polypeptides of the invention are useful as antigenic material. Such material can be "antigenic" and/or "immunogenic". Generally, "antigenic" is taken to mean that the protein or polypeptide is capable of being used to raise antibodies or indeed is capable of inducing an antibody response in a subject. "Immunogenic" is taken to mean that the protein or polypeptide is capable of

eliciting a protective immune response in a subject. Thus, in the latter case, the protein or polypeptide may be capable of not only generating an antibody response and in addition non-antibody based immune responses.

The skilled person will appreciate that homologues or derivatives of the proteins or polypeptides of the invention will also find use in the context of the present invention, ie as antigenic/immunogenic material. Thus, for instance proteins or polypeptides which include one or more additions, deletions, substitutions or the like are encompassed by the present invention. In addition, it may be possible to replace one amino acid with another of similar "type". For instance replacing one hydrophobic amino acid with another. One can use a program such as the CLUSTAL program to compare amino acid sequences. This program compares amino acid sequences and finds the optimal alignment by inserting spaces in either sequence as appropriate. It is possible to calculate amino acid identity or similarity (identity plus conservation of amino acid type) for an optimal alignment. A program like BLASTx will align the longest stretch of similar sequences and assign a value to the fit. It is thus possible to obtain a comparison where several regions of similarity are found, each having a different score. Both types of analysis are contemplated in the present invention.

In the case of homologues and derivatives, the degree of identity with a protein or polypeptide as described herein is less important than that the homologue or derivative should retain its antigenicity or immunogenicity to streptoccocus pneumoniae. However, suitably, homologues or derivatives having at least 60% similarity (as discussed above) with the proteins or polypeptides described herein are provided.

Preferably, homologues or derivatives having at least 70% similarity, more preferably at least 80% similarity are provided. Most preferably, homologues or derivatives having at least 90% or even 95% similarity are provided.

In an alternative approach, the homologues or derivatives could be fusion proteins, incorporating moieties which render purification easier, for example by effectively tagging the desired protein or polypeptide. It may be necessary to remove the "tag" or it may be the case that the fusion protein itself retains sufficient antigenicity to be useful.

In an additional aspect of the invention there are provided antigenic fragments of the proteins or polypeptides of the invention, or of homologues or derivatives thereof.

For fragments of the proteins or polypeptides described herein, or of homologues or derivatives thereof, the situation is slightly different. It is well known that is possible to screen an antigenic protein or polypeptide to identify epitopic regions, ie those regions which are responsible for the protein or polypeptide's antigenicity or immunogenicity. Methods for carrying out such screening are well known in the art. Thus, the fragments of the present invention should include one or more such epitopic regions or be sufficiently similar to such regions to retain their antigenic/immunogenic properties. Thus, for fragments according to the present invention the degree of identity is perhaps irrelevant, since they may be 100% identical to a particular part of a protein or polypeptide, homologue or derivative as described herein. The key issue, once again, is that the fragment retains the antigenic/immunogenic properties.

Thus, what is important for homologues, derivatives and fragments is that they possess at least a degree of the antigenicity/immunogenicity of the protein or polypeptide from which they are derived.

Gene cloning techniques may be used to provide a protein of the invention in substantially pure form. These techniques are disclosed, for example, in J. Sambrook *et al Molecular Cloning* 2nd Edition, Cold Spring Harbor Laboratory Press (1989). Thus, in a fourth aspect, the present invention provides a nucleic acid molecule comprising or consisting of a sequence which is:

(i) any of the DNA sequences set out in Table 1 or their RNA equivalents;

(ii) a sequence which is complementary to any of the sequences of (i);

(iii) a sequence which codes for the same protein or polypeptide, as those sequences of (i) or (ii);

(iv) a sequence which is has substantial identity with any of those of (i), (ii) and (iii);

(v) a sequence which codes for a homologue, derivative or fragment of a protein as defined in Table 1.

In a fifth aspect the present invention provides a nucleic acid molecule comprising or consisting of a sequence which is:

(i) any of the DNA sequences set out in Table 4 or their RNA equivalents;

(ii) a sequence which is complementary to any of the sequences of (i);

(iii) a sequence which codes for the same protein or polypeptide, as those sequences of (i) or (ii);

(iv) a sequence which is has substantial identity with any of those of (i), (ii) and (iii);

(v)a sequence which codes for a homologue, derivative or fragment of a protein as defined in Table 4.

The nucleic acid molecules of the invention may include a plurality of such sequences, and/or fragments. The skilled person will appreciate that the present invention can include novel variants of those particular novel nucleic acid molecules which are exemplified herein. Such variants are encompassed by the present invention. These may occur in nature, for example because of strain variation. For example, additions, substitutions and/or deletions are included. In addition, and particularly when utilising microbial expression systems, one may wish to engineer the nucleic acid sequence by making use of known preferred codon usage in the particular organism being used for expression. Thus, synthetic or non-naturally occurring variants are also included within the scope of the invention.

The term "RNA equivalent" when used above indicates that a given RNA molecule has a sequence which is complementary to that of a given DNA molecule (allowing for the fact that in RNA "U" replaces "T" in the genetic code).

When comparing nucleic acid sequences for the purposes of determining the degree of homology or identity one can use programs such as BESTFIT and GAP (both from the Wisconsin Genetics Computer Group (GCG) software package) BESTFIT, for example, compares two sequences and produces an optimal alignment of the most

similar segments. GAP enables sequences to be aligned along their whole length and finds the optimal alignment by inserting spaces in either sequence as appropriate. Suitably, in the context of the present invention compare when discussing identity of nucleic acid sequences, the comparison is made by alignment of the sequences along their whole length.

Preferably, sequences which have substantial identity have at least 50% sequence identity, desirably at least 75% sequence identity and more desirably at least 90 or at least 95% sequence identity with said sequences. In some cases the sequence identity may be 99% or above.

Desirably, the term "substantial identity" indicates that said sequence has a greater degree of identity with any of the sequences described herein than with prior art nucleic acid sequences.

It should however be noted that where a nucleic acid sequence of the present invention codes for at least part of a novel gene product the present invention includes within its scope all possible sequence coding for the gene product or for a novel part thereof.

The nucleic acid molecule may be in isolated or recombinant form. It may be incorporated into a vector and the vector may be incorporated into a host. Such vectors and suitable hosts form yet further aspects of the present invention.

Therefore, for example, by using probes based upon the nucleic acid sequences provided herein, genes in *Streptococcus pneumoniae* can be identified. They can then be excised using restriction enzymes and cloned into a vector. The vector can be introduced into a suitable host for expression.

Nucleic acid molecules of the present invention may be obtained from *S.pneumoniae* by the use of appropriate probes complementary to part of the sequences of the nucleic acid molecules. Restriction enzymes or sonication techniques can be used to obtain appropriately sized fragments for probing.

Alternatively PCR techniques may be used to amplify a desired nucleic acid sequence. Thus the sequence data provided herein can be used to design two primers for use in PCR so that a desired sequence, including whole genes or fragments thereof, can be targeted and then amplified to a high degree. One primer will normally show a high degree of specificity for a first sequence located on one strand of a DNA molecule, and the other primer will normally show a high degree of specificity for a second sequence located on the complementary strand of the DNA sequence and being spaced from the complementary sequence to the first sequence.

Typically primers will be at least 15-25 nucleotides long.

As a further alternative chemical synthesis may be used. This may be automated. Relatively short sequences may be chemically synthesised and ligated together to provide a longer sequence.

In yet a further aspect the present invention provides an immunogenic/antigenic composition comprising one or more proteins or polypeptides selected from those whose sequences are shown in Tables 2-4, or homologues or derivatives thereof, and/or fragments of any of these. In preferred embodiments, the immunogenic/antigenic composition is a vaccine or is for use in a diagnostic assay.

In the case of vaccines suitable additional excipients, diluents, adjuvants or the like may be included. Numerous examples of these are well known in the art.

It is also possible to utilise the nucleic acid sequences shown in Table 1 in the preparation of so-called DNA vaccines. Thus, the invention also provides a vaccine composition comprising one or more nucleic acid sequences as defined herein. The use of such DNA vaccines is described in the art. See for instance, Donnelly *et al* , *Ann. Rev. Immunol.,* **15**:617-648 (1997).

As already discussed herein the proteins or polypeptides described herein, their homologues or derivatives, and/or fragments of any of these, can be used in methods of detecting/diagnosing *S.pneumoniae*. Such methods can be based on the detection of antibodies against such proteins which may be present in a subject. Therefore the present invention provides a method for the detection/diagnosis of *S.pneumoniae* which comprises the step of bringing into contact a sample to be tested with at least one protein, or homologue, derivative or fragment thereof, as described herein. Suitably, the sample is a biological sample, such as a tissue sample or a sample of blood or saliva obtained from a subject to be tested.

In an alternative approach, the proteins described herein, or homologues, derivatives and/or fragments thereof, can be used to raise antibodies, which in turn can be used to detect the antigens, and hence *S.pneumoniae*. Such antibodies form another aspect of the invention. Antibodies within the scope of the present invention may be monoclonal or polyclonal.

Polyclonal antibodies can be raised by stimulating their production in a suitable animal host (e.g. a mouse, rat, guinea pig, rabbit, sheep, goat or monkey) when a protein as described herein, or a homologue, derivative or fragment thereof, is injected into the animal. If desired, an adjuvant may be administered together with the protein. Well-known adjuvants include Freund's adjuvant (complete and incomplete) and aluminium

hydroxide. The antibodies can then be purified by virtue of their binding to a protein as described herein.

Monoclonal antibodies can be produced from hybridomas. These can be formed by fusing myeloma cells and spleen cells which produce the desired antibody in order to form an immortal cell line. Thus the well-known Kohler & Milstein technique (*Nature* **256** (1975)) or subsequent variations upon this technique can be used.

Techniques for producing monoclonal and polyclonal antibodies that bind to a particular polypeptide/protein are now well developed in the art. They are discussed in standard immunology textbooks, for example in Roitt *et al*, *Immunology* second edition (1989), Churchill Livingstone, London.

In addition to whole antibodies, the present invention includes derivatives thereof which are capable of binding to proteins etc as described herein. Thus the present invention includes antibody fragments and synthetic constructs. Examples of antibody fragments and synthetic constructs are given by Dougall *et al* in *Tibtech* **12** 372-379 (September 1994).

Antibody fragments include, for example, Fab, $F(ab')_2$ and Fv fragments. Fab fragments (These are discussed in Roitt *et al* [*supra*]). Fv fragments can be modified to produce a synthetic construct known as a single chain Fv (scFv) molecule. This includes a peptide linker covalently joining V_h and V_l regions, which contributes to the stability of the molecule. Other synthetic constructs that can be used include CDR peptides. These are synthetic peptides comprising antigen-binding determinants. Peptide mimetics may also be used. These molecules are usually conformationally restricted organic rings that mimic the structure of a CDR loop and that include antigen-interactive side chains.

Synthetic constructs include chimaeric molecules. Thus, for example, humanised (or primatised) antibodies or derivatives thereof are within the scope of the present invention. An example of a humanised antibody is an antibody having human framework regions, but rodent hypervariable regions. Ways of producing chimaeric antibodies are discussed for example by Morrison *et al* in PNAS, **81,** 6851-6855 (1984) and by Takeda *et al* in Nature. **314,** 452-454 (1985).

Synthetic constructs also include molecules comprising an additional moiety that provides the molecule with some desirable property in addition to antigen binding. For example the moiety may be a label (e.g. a fluorescent or radioactive label). Alternatively, it may be a pharmaceutically active agent.

Antibodies, or derivatives thereof, find use in detection/diagnosis of *S.pneumoniae*. Thus, in another aspect the present invention provides a method for the detection/diagnosis of *S.pneumoniae* which comprises the step of bringing into contact a sample to be tested and antibodies capable of binding to one or more proteins described herein, or to homologues, derivatives and/or fragments thereof.

In addition, so-called "Affibodies" may be utilised. These are binding proteins selected from combinatorial libraries of an alpha-helical bacterial receptor domain (Nord *et al* ,) Thus, Small protein domains, capable of specific binding to different target proteins can be selected using combinatorial approaches.

It will also be clear that the nucleic acid sequences described herein may be used to detect/diagnose *S.pneumoniae*. Thus, in yet a further aspect, the present invention provides a method for the detection/diagnosis of *S.pneumoniae* which comprises the

step of bringing into contact a sample to be tested with at least one nucleic acid sequence as described herein. Suitably, the sample is a biological sample, such as a tissue sample or a sample of blood or saliva obtained from a subject to be tested. Such samples may be pre-treated before being used in the methods of the invention. Trhus, for example, a sample may be treated to extract DNA. Then, DNA probes based on the nucleic acid sequences described herein (ie usually fragments of such sequences) may be used to detect nucleic acid from *S.pneumoniae*.

In additional aspects, the present invention provides:

(a) a method of vaccinating a subject against *S.pneumoniae* which comprises the step of administering to a subject a protein or polypeptide of the invention, or a derivative, homologue or fragment thereof, or an immunogenic composition of the invention;

(b) a method of vaccinating a subject against *S.pneumoniae* which comprises the step of administering to a subject a nucleic acid molecule as defined herein;

(c) a method for the prophylaxis or treatment of *S.pneumoniae* infection which comprises the step of administering to a subject a protein or polypeptide of the invention, or a derivative, homologue or fragment thereof, or an immunogenic composition of the invention;

(d) a method for the prophylaxis or treatment of *S.pneumoniae* infection which comprises the step of administering to a subject a nucleic acid molecule as defined herein;

(e) a kit for use in detecting/diagnosing *S.pneumoniae* infection comprising one

or more proteins or polypeptides of the invention, or homologues, derivatives or fragments thereof, or an antigenic composition of the invention; and

(f) a kit for use in detecting/diagnosing *S.pneumoniae* infection comprising one or more nucleic acid molecules as defined herein.

Given that we have identified a group of important proteins, such proteins are potential targets for anti-microbial therapy. It is necessary, however, to determine whether each individual protein is essential for the organism's viability. Thus, the present invention also provides a method of determining whether a protein or polypeptide as described herein represents a potential anti-microbial target which comprises inactivating said protein and determining whether *S.pneumoniae* is still viable, *in vitro* or *in vivo*.

A suitable method for inactivating the protein is to effect selected gene knockouts, ie prevent expression of the protein and determine whether this results in a lethal change. Suitable methods for carrying out such gene knockouts are described in Li *et al*, *P.N.A.S.*, **94**:13251-13256 (1997).

In a final aspect the present invention provides the use of an agent capable of antagonising, inhibiting or otherwise interfering with the function or expression of a protein or polypeptide of the invention in the manufacture of a medicament for use in the treatment or prophylaxis of *S.pneumoniae* infection.

The invention will now be described with reference to the following examples, which should not be construed as in any way limiting the invention. The examples refer to the figures in which:

Figure 1: shows the results of various DNA vaccine trials; and

Figure 2: shows the results of further DNA vaccine trials.

EXAMPLE 1

The Genome sequencing of *Streptococcus pneumoniae* type 4 is in progress at the

Institute for Genomic Research (TIGR, Rockville, MD, USA). Up to now, the whole sequence has not been completed or published. On 21st November 1997, the TIGR centre released some DNA sequences as contigs which are not accurate reflections of the finished sequence. These contigs can be downloaded from their Webster (www@tigr.org). We downloaded these contigs and created a local database using the application GCGToBLAST (Wisconsin Package Version 9.1, Genetics Computer Group (GCG), Madison, USA). This database can be searched with the FastA and TfastA procedures (using the method of Pearson and Lipman (*PNAS USA*, **85**:2444-2448 (1988)).

Using FastA and TfastA procedures, the local pneumococcus database was searched for putative leader sequence or anchor sequence features. Relevant sequences were used to interrogate for comparative novel sequences. These were:

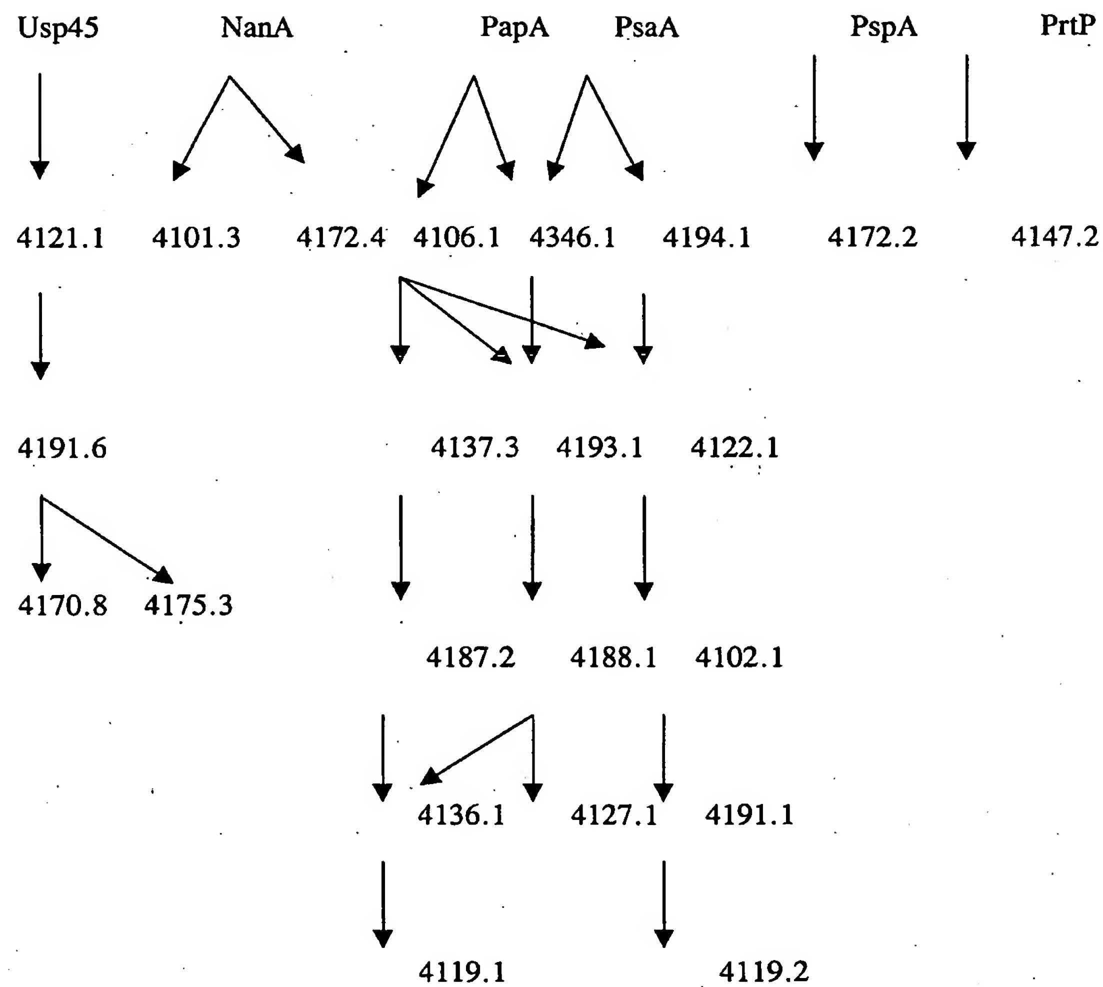
(i) already described leader sequences of *Streptococcus pneumoniae* (from proteins NanA, NanB, LytA, PapA, pcpA, PsaA and PspA);

(ii) the leader sequence of Usp45, a secreted protein from *Lactococcus lactis*;

(iii) new hypothetical leader sequences derived from the searches in (i) and (ii);

(iv) the anchor motif LPxTG, a feature common to many Gram-positive bacteria surface proteins which are anchored by a mechanism involving the Sortase complex proteins.

Provided below is an example of this approach, with reference to the sequences derived from the database (see table 1).



The protein leader sequences of different known exported proteins were used as a starting point for a search of the local pneumococcus database described above. The hypothetical proteins found with this search were then submitted to a Blast search in general databases such as EMBL, Swissprot etc. Proteins remaining unknown in the pneumococcus are kept and annotated. Then the search is performed again using the new potential protein leader sequence as a probe, using the TfastA procedure.

Example 2: DNA vaccine trials

pcDNA3.1+ as a DNA vaccine vector

pcDNA3.1+

The vector chosen for use as a DNA vaccine vector was pcDNA3.1 (Invitrogen) (actually pcDNA3.1+, the forward orientation was used in all cases but may be referred to as pcDNA3.1 here on). This vector has been widely and successfully employed as a host vector to test vaccine candidate genes to give protection against pathogens in the literature (Zhang, *et al.*, Kurar and Splitter, Anderson *et al.*). The vector was designed for high-level stable and non-replicative transient expression in mammalian cells. pcDNA3.1 contains the ColE1 origin of replication which allows convenient high-copy number replication and growth in *E. coli*. This in turn allows rapid and efficient cloning and testing of many genes. The pcDNA3.1 vector has a large number of cloning sites and also contains the gene encoding ampicillin resistance to aid in cloning selection and the human cytomegalovirus (CMV) immediate-early promoter/enhancer which permits efficient, high-level expression of the recombinant protein. The CMV promoter is a strong viral promoter in a wide range of cell types including both muscle and immune (antigen presenting) cells. This is important for optimal immune response as it remains unknown as to which cells types are most important in generating a protective response *in vivo*. A T7 promoter upstream of the multiple cloning site affords efficient expression of the modified insert of interest and which allows *in vitro* transcription of a cloned gene in the sense orientation.

Zhang, D., Yang, X., Berry, J. Shen, C., McClarty, G. and Brunham, R.C. (1997) "DNA vaccination with the major outer-membrane protein genes induces acquired immunity to *Chlamydia trachomatis* (mouse pneumonitis) infection". *Infection and Immunity*, **176**, 1035-40.

Kurar, E. and Splitter, G.A. (1997) "Nucleic acid vaccination of *Brucella abortus* ribosomal *L7/L12* gene elicits immune response". *Vaccine*, **15**, 1851-57.

Anderson, R., Gao, X.-M., Papakonstantinopoulou, A., Roberts, M. and Dougan, G. (1996) "Immune response in mice following immunisation with DNA encoding fragment C of tetanus toxin". *Infection and Immunity*, **64**, 3168-3173.

Preparation of DNA vaccines

Oligonucleotide primers were designed for each individual gene of interest derived using the LEEP system. Each gene was examined thoroughly, and where possible,

primers were designed such that they targeted that portion of the gene thought to encode only the mature portion of the gene protein. It was hoped that expressing those sequences that encode only the mature portion of a target gene protein, would facilitate its correct folding when expressed in mammalian cells. For example, in the majority of cases primers were designed such that putative N-terminal signal peptide sequences would not be included in the final amplification product to be cloned into the pcDNA3.1 expression vector. The signal peptide directs the polypeptide precursor to the cell membrane via the protein export pathway where it is normally cleaved off by signal peptidase I (or signal peptidase II if a lipoprotein). Hence the signal peptide does not make up any part of the mature protein whether it be displayed on the surface of the bacteria surface or secreted. Where a N-terminal leader peptide sequence was not immediately obvious, primers were designed to target the whole of the gene sequence for cloning and ultimately, expression in pcDNA3.1.

Having said that, however, other additional features of proteins may also affect the expression and presentation of a soluble protein. DNA sequences encoding such features in the genes encoding the proteins of interest were excluded during the design of oligonucleotides. These features included:

1. LPXTG cell wall anchoring motifs.
2. LXXC ipoprotein attachment sites.
3. Hydrophobic C-terminal domain.
4. Where no N-terminal signal peptide or LXXC was present the start codon was excluded.
5. Where no hydrophobic C-terminal domain or LPXTG motif was present the stop codon was removed.

Appropriate PCR primers were designed for each gene of interest and any and all of the regions encoding the above features was removed from the gene when designing these primers. The primers were designed with the appropriate enzyme restriction site followed by a conserved Kozak nucleotide sequence (in all cases) GCCACC was used. The Kozak sequence facilitates the recognition of initiator sequences by eukaryotic ribosomes) and an ATG start codon upstream of the insert of the gene of interest. For example the forward primer using a BamH1 site the primer would begin GCGGGATCCGCCACCATG followed by a small section of the 5' end of the gene of interest. The reverse primer was designed to be compatible with the forward primer and with a Not1 restriction site at the 5' end in all cases (this site is TTGCGGCCGC).

<u>PCR primers</u>

The following PCR primers were designed and used to amplify the truncated genes of interest.

ID210

Forward Primer 5' CGGATCCGCCACCATGTCTTCTAATGAATCTGCCGATG 3'
Reverse Primer 5' TTGCGGCCGCCTGTTTAGATTGGATATCTGTAAAGACTT 3'

4172.5

Forward Primer 5' CGCGGATCCGCCACCATGGATTTTCCTTCAAATTTGGAGG 3'
Reverse Primer 5' TTGCGGCCGCACCGTACTGGCTGCTGACT 3'

ID211

Forward Primer 5' CGGATCCGCCACCATGAGTGAGATCAAAATTATTAACGC 3'
Reverse Primer 5' TTGCGGCCGCCGTTCCATGGTTGACTCCT 3'

4197.4

Forward Primer 5' CGCGGATCCGCCACCATGTGGGACATATTGGTGGAAAC 3'
Reverse Primer 5' TTGCGGCCGCTTCACTTGAGCAAACTGAATCC 3'

4122.1

Forward Primer 5' CGCGGATCCGCCACCATGTCACAAGAAAAAACAAAAAATGAA 3'
Reverse Primer 5' TTGCGGCCGCATCGACGTAGTCTCCGCC 3'

4126.7

Forward Primer 5' CGCGGATCCGCCACCATGCTGGTTGGAACTTTCTACTATCAAT 3'
Reverse Primer 5' TTGCGGCCGCAACTTTCGTCCCTTTTTGG 3'

4188.11

Forward Primer 5' CGCGGATCCGCCACCATGGGCAATTCTGGCGGAA 3'
Reverse Primer 5' TTGCGGCCGCTTGTTTCATAGCTTTTTGATTGTT 3'

ID209

Forward Primer 5' CGCGGATCCGCCACCATGCTATTGATACGAAATGCAGGG 3'
Reverse Primer 5' TTGCGGCCGCAACATAATCTAGTAAATAAGCGTAGCC 3'

ID215

Forward Primer 5' CGCGGATCCGCCACCATGACGGCGACGAATTTTC 3'
Reverse Primer 5' TTGCGGCCGCTTAATTCGTTTTTGAACTAGTTGCT 3'

4170.4

Forward Primer 5' CGCGGATCCGCCACCATGGCTGTTTTTCTTCGCTATCATG 3'
Reverse Primer 5' TTGCGGCCGCTTTCTTCAACAAACCTTGTTCTTG 3'

4193.1

Forward Primer 5' CGCGGATCCGCCACCATGGGTAACCGCTCTTCTCGTAAC 3'
Reverse Primer 5' TTGCGGCCGCGCTTCCATCAAGGATTTTAGC 3'

Cloning

The insert along with the flanking features described above was amplified using PCR against a template of genomic DNA isolated from type 4 *S. pneumoniae* strain 11886 obtained from the National Collection of Type Cultures. The PCR product was cut with the appropriate restriction enzymes and cloned in to the multiple cloning site of pcDNA3.1 using conventional molecular biological techniques. Suitably mapped clones of the genes of interested were cultured and the plasmids isolated on a large scale (>1.5 mg) using Plasmid Mega Kits (Qiagen). Successful cloning and maintenance of genes was confirmed by restriction mapping and sequencing ~700 base pairs through the 5' cloning junction of each large scale preparation of each construct.

Strain validation

A strain of type 4 was used in cloning and challenge methods which is the strain from which the *S. pneumoniae* genome was sequenced. A freeze dried ampoule of a homogeneous laboratory strain of type 4 *S. pneumoniae* strain NCTC 11886 was obtained from the National Collection of Type Strains. The ampoule was opened and the cultured re suspended with 0.5 ml of tryptic soy broth (0.5% glucose, 5% blood). The suspension was subcultured into 10 ml tryptic soy broth (0.5% glucose, 5% blood) and incubated statically overnight at 37°C. This culture was streaked on to 5% blood agar plates to check for contaminants and confirm viability and on to blood agar slopes and the rest of the culture was used to make 20% glycerol stocks. The slopes were sent to the Public Health Laboratory Service where the type 4 serotype was confirmed.

A glycerol stock of NCTC 11886 was streaked on a 5% blood agar plate and incubated overnight in a CO2 gas jar at 37°C. Fresh streaks were made and optochin sensitivity was confirmed.

<u>Pneumococcal challenge</u>

A standard inoculum of type 4 *S. pneumoniae* was prepared and frozen down by passaging a culture of pneumococcus 1x through mice, harvesting from the blood of infected animals, and grown up to a predetermined viable count of around 10^9 cfu/ml in broth before freezing down. The preparation is set out below as per the flow chart.

Streak pneumococcal culture and confirm identity

|
V

Grow over-night culture from 4-5 colonies on plate above

|
V

Animal passage pneumococcal culture
(i.p. injection of cardiac bleed to harvest)

|
V

Grow over-night culture from animal passaged pneumococcus

|
V

Grow day culture (to pre-determined optical density) from over-night of animal passage and freeze down at -70°C - This is standard minimum

|
V

Thaw one aliquot of standard inoculum to viable count

|
V

Use standard inoculum to determine effective dose (called Virulence Testing)

|
V

All subsequent challenges - use standard inoculum to effective dose

An aliquot of standard inoculum was diluted 500x in PBS and used to inoculate the mice.

Mice were lightly anaesthetised using halothane and then a dose of 1.4 x 10^5 cfu of pneumococcus was applied to the nose of each mouse. The uptake was facilitated by the normal breathing of the mouse, which was left to recover on its back.

***S. pneumoniae* vaccine trials**

Vaccine trials in mice were carried out by the administration of DNA to 6 week old CBA/ca mice (Harlan, UK). Mice to be vaccinated were divided into groups of six and each group was immunised with recombinant pcDNA3.1+ plasmid DNA containing a specific target-gene sequence of interest. A total of 100 μg of DNA in Dulbecco's PBS (Sigma) was injected intramuscularly into the tibialis anterior muscle of both legs (50 μl in each leg). A boost was carried using the same procedure 4 weeks later. For comparison, control groups were included in all vaccine trials. These control groups were either unvaccinated animals or those administered with non-recombinant pcDNA3.1+ DNA (sham vaccinated) only, using the same time course described above. 3 weeks after the second immunisation, all mice groups were challenged intra-nasally with a lethal dose of *S. pneumoniae*

serotype 4 (strain NCTC 11886). The number of bacteria administered was monitored by plating serial dilutions of the inoculum on 5% blood agar plates. A problem with intranasal immunisations is that in some mice the inoculum bubbles out of the nostrils, this has been noted in results table and taken account of in calculations. A less obvious problem is that a certain amount of the inoculum for each mouse may be swallowed. It is assumed that this amount will be the same for each mouse and will average out over the course of innoculations. However, the sample sizes that have been used are small and this problem may have significant effects in some experiments. All mice remaining after the challenge were killed 3 or 4 days after infection. During the infection process, challenged mice were monitored for the development of symptoms associated with the onset of *S. pneumoniae* induced-disease. Typical symptoms in an appropriate order included piloerection, an increasingly hunched posture, discharge from eyes, increased lethargy and reluctance to move. The latter symptoms usually coincided with the development of a moribund state at which stage the mice were culled to prevent further suffering. These mice were deemed to be very close to death, and the time of culling was used to determine a survival time for statistical analysis. Where mice were found dead, the survival time was taken as the last time point when the mouse was monitored alive.

Interpretation of Results

A positive result was taken as any DNA sequence that was cloned and used in challenge experiments as described above which gave protection against that challenge. Protection was taken as those DNA sequences that gave statistically significant protection (to a 95% confidence level ($p<0.05$)) and also those which were marginal or close to significant using Mann-Whitney or which show some protective features for example there were one or more outlying mice or because the time to the first death was prolonged. It is acceptable to allow marginal or non-significant results to be considered as potential positives when it is considered that the clarity of some of the results may be clouded by the problems associated with the administration of intranasal infections.

Results for vaccine trials 2, 7 and 8 (see figure 1)

Mouse number	Mean survival times (hours)								
	Unvacc control (2)	ID210 (2)	Unvacc control (7)	4172.5 (7)	Unvacc control (8)	ID211 (8)	4197.4 (8)	4122.1 (8)	4126.7 (8)
1	49.0	55.0	59.6	72.6	45.1	102.3T	60.1	50.6	60.0
2	51.0	46.5	47.2	67.9	50.8	55.5	54.9	77.2	60.0
3	49.0	49.0	59.6	54.4	60.4	60.6*	68.4	60.3	54.8
4	55.0	59.0	70.9	75.3	55.2	45.3	60.1	50.6	52.6
5	49.0	55.0	68.6*	70.9	45.1	55.5	54.9	50.6*	54.8
6	49.0	49.0	76.0	75.3	45.1	102.3T	52.7	44.9	60
Mean	**50.3**	**52.3**	**63.6**	**69.4**	**50.2**	**70.2**	**58.5**	**55.7**	**57.0**
sd	**2.4**	**4.8**	**10.3**	**7.9**	**6.4**	**25.3**	**5.7**	**11.6**	**3.4**
p value 1	-	**0.3333**	-	**0.2104**	-	**0.0215**	**0.0621**	**0.4038**	**0.0833**

* - bubbled when dosed so may not have received full inoculum.

T - terminated at end of experiment having no symptoms of infection.

Numbers in brackets - survival times disregarded assuming incomplete dosing

p value 1 refers to significance tests compared to unvaccinated controls

Statistical Analyses.

Trial 2 - The group vaccinated with ID210 also had a longer mean survival time than the unvaccinated controls but the results are not statistically significant.

Trial 7 - The group vaccinated with 4172.5 showed much greater survival times than unvaccinated controls although the differences were not statistically significant.

Trial 8 - The group vaccinated with ID211 survived significantly longer than unvaccinated controls. 4197.4, 4122.1 and 4126.7 vaccinated groups showed longer mean survival times than the unvaccinated group but the results were not statistically significant. The 4197.4 and 4126.7 groups also showed a prolonged time to the first death and the 4122.1 group showed 1 outlying result.

Results of pneumococcal challenge DNA vaccination trials 9-11
(see figure 2)

Mouse number	Mean survival times (hours)									
	Unvacc control (9)	4188.1 1 (9)	ID209 (9)	Unvacc control (10)	pcDNA3.1 + (10)	ID215 (10)	4170. 4 (10)	Unvacc control (11)	pcDNA3.1 + (11)	4193.1 (11)
1	(98.5)T	69.4	60.2	68.4	58.6	79.2	68.1	60.0	53.2	54.8
2	53.4	53.7	60.2	59.0	58.6	54.2	58.6	50.0	50.4	54.8
3	53.4	51.2	60.2	59.0	50.8	(103.2)*T	50.9	60.0	55.4	68.7*
4	53.4	75.0	(98.0)*T	45.1*	58.6	58.8	72.1	55.0	60.6	54.8
5	70.8	51.2	60.2	68.4	46.5	68.3	68.1	60.0	50.4	68.7
6	53.4	61.2	52.9	59.0	48.9	58.8	54.0	50.0	60.6	68.7*
Mean	56.9	60.3	58.8	59.8	53.6	63.9	62.0	55.8	55.1	61.7
Sd	7.8	10.0	3.3	8.5	5.6	10.0	8.7	5.0	4.6	7.6
p value 1	-	0.3894	0.2519	-	0.0307	<30.0	<39. 0	-	-	0.1837
p value 2	-	-	-	-	-	0.0168	0.031 6	-	-	0.0829

* - bubbled when dosed so may not have received full inoculum.

T - terminated at end of experiment having no symptoms of infection.

Numbers in brackets - survival times disregarded assuming incomplete dosing

p value 1 refers to significance tests compared to unvaccinated controls

p value 2 refers to significance tests compared to pcDNA3.1+ vaccinated controls

Statistical Analyses.

Trial 9 - Although not statistically significant the groups vaccinated with 4188.11 and ID209 did have noticeably higher mean survival times than unvaccinated controls.

Trial 10 - The unvaccinated control group survived for a significantly longer period than the pcDNA3.1+ vaccinated group. The groups vaccinated with ID215 and 4170.4 showed statistically significant longer survival times compared to the sham vaccinated group ($p=0.0168$ and 0.0316) but not compared to the unvaccinated group.

Trial 11 - The group vaccinated with 4193.1 was the most promising and survived an average of 6.5 hours longer than the pcDNA3.1+ vaccinated group and 6 hours longer than the unvaccinated group although the results were not statistically significant.

Table 1

4101.1
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4101.3
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4101.5
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4102.1

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4106.1
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4106.4
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4106.6
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4106.7
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4106.8
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4106.10
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4107.1
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4107.2
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4107.3
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4109.1

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4110.2

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4112.2

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4113.1

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4117.1

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4119.2
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4119.3
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4119.4
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4120.1
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4121.2
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4122.1
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4125.6
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4125.7
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4125.10
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4126.1
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4126.7
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4127.4
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4127.5
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4128.1
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4128.2
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4129.2
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4133.1
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4135.2
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4136.2

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4137.2
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4138.1
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4139.1
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4139.5
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4139.8
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4141.1
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4142.3
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4142.4
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4142.5
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4144.1
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4144.2
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4144.3
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4146.1
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4146.2
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4147.1
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4147.2
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TAACAGAAGAATAA

4147.3
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GTGA

4149.1
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4149.2
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TGA

4149.3
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4152.2
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4154.1
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4154.2
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4155.1

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TGA

4156.1

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4156.4

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4157.2

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4158.1
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4158.2
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4158.3
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4158.4
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4158.5
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4158.6
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4158.7
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4161.1
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4161.2
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4162.1
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4162.2
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4164.2
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4164.3
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4166.2
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4166.3
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4169.1

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CATTAA

4169.3

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4169.4
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4169.6
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4170.3
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4170.4
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4170.5
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4170.6
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4170.8
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4171.1
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4172.1
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4172.2
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4172.3
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4172.4
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4172.5

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4172.6

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4174.1

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4175.2

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4175.3

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4174.4

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4175.5

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4175.6

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4176.1

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4178.2
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4179.1
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4179.2
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4179.3
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4179.4
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4179.6
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4179.7
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4179.8
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4179.9
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CGGAGATGAATAA

4179.12
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4181.1
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4181.2
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4183.1
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4183.5
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4183.6
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4183.7
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4183.8
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4185.3

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TAA

4186.1

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4186.2

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4187.2

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4188.1
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4188.2
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4188.5
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4188.10
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4188.11
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4188.12
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4191.1
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4191.2
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4191.3
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4191.4
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4191.5
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4191.6
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4192.3
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4193.1
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4193.3
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4194.1
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4194.4
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4196.2
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4197.1
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4197.4
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4211.2·
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4211.3
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4211.4
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4213.1
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4213.2

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4224.1

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4252.1

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4252.2

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4256.2
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4263.1
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4346.1
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4346.2
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4346.3
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GAAATAA

Table 2

MEELVTLDCLFIDRTKIEANANKYSFVWKKTTEKFSAKLQEQIQVYFQEEITPLLIKYAMFDKKQKRGYKESAKNLANW
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DGVYMDSQVYVNGQLVGHYPNGYNQFSYDITKYLQKDGRENVIAVHAVNKQPSSRWYSGSGIYRDVTLQVTDKVHV
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TZ

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VISVTQELVDAKGANLGVLRLDISYETLEAYLNQLQLGQQGFAFIINENHEFVYHPQHTVYSSSSKMEAMKPYIDTGQG
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GAQNLRAKEVGAYELREVTRQFNAMLDQIDQLMVAIRSQEETTRQYQLQALSSQINPHFLYNTLDTIIWMAEFHDSQR
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YMYVMSFLSVPAYLFILLGFIFPNHSGLAALSKEFMVGKPLWVYVVYISVLFLFSIIFAFVTMNGEEIADRMKKSGEYIYGI
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MSSLSDQELVAKTVEFRQRLSEGESLDDILVEAFAVVREADKRILGMFPYDVQVMGAIVMHYGNVAEMNTGEGKTLT
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KLSPETRAMASITYQSLFKMFNKISGMTGTGKVAEKEFIETYNMSVVRIPTNRPRQRIDYPDNLYITLPEKVYASLEYIKQ
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GGLIVIGTERMESQRIDLQIRGRSGRQGDPGMSKFFVSLEDDVIKKFGPSWVHKKYKDYQVQDMTQPEVLKGRKYRKL
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ISFHVKEVPDYIDVTDKTAVRSFMKQVIDKELSEKKELLNQHDLYEQFLRLSLLKAIDDNWVEQVDYLQQLSMAIGGQS
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NCLRVSGVTAFFTRSSATNIPVNMKLCHDLGLNPDTYSVSIPLGSTINMAGVAITINLLTLAAVNTLGIPVDFATAFVLSV VAAISSCDASGIAGGSLLLIPVACSLFGISNDIAIQIVGVGFVIGVIQDSCETALNSSTDVLFTAVAEYAATRKKZ

MSISQRTTKLILATCLACLLAYFLNLSSAVSAGIIALLSLSDTRRSTLKLARNRLFSMLLALAIGVLAFHLSGFHIWSLGLY LAFYVPLAYKMGWEIGITPSTVLVSHLLVQESTSPDLLVNEFLLFAIGTGFALLVNLYMPSREEEIQHYHTLVEEKLKDI LQRFKYYLSRGDGRNRAQLVAELDTLLKEALRLVYLDHSDHLFHQTDYHIHYFEMRQRQSRILRNMAQQINTCHLAAS ESLILAQLFSKIAGQLSQTNPASDLLDEIERYLEVFRNRSLPKTREEFETRATLLQLLREAKTFIQVKVDFYQKYRQZ

MEIMSLAIAVFAVIIGLVIGYVSISAKMKSSQEAAELMLLNAEQEATNLRGQAEREADLLVNEAKRESKSLKKEALLEAK EEARKYREEVDAEFKSERQELKQIESRLTERATSLDRKDDNLTSKEQTLEQKEQSISDRAKNLDAREEQLEEVERQKEAE LERIGALSQAEARDIILAQTEENLTREIASRIREAEQEVKERSDKMAKDILVQAMQRIAGEYVAESTNSTVHLPDDTMKG RIIGREGRNIRTFESLTGVDVIIDDTPEVVTLSGFDPIRREIARMTMEMLLKDGRIHPARIEELVEKNRQEIDNKIREYGEA AAYEIGAPNLHPDLMKIMGRLQFRTSYGQNVLRHSIEVAKLAGIMASELGENAALARRAGFLHDIGKAIDHEVEGSHVE IGMELARKYKEPPVVVNTIASHHGDVEAESVIAVIVAAADALSAARPGARSESLESYIKRLHDLEEIANGFEGVQTSFAL QAGREIRIMVNPGKIKDDKVTILAHKVRKKIENNLDYPGNIKVTVIRELRAVDYAKZ

MMLKPSIDTLLDKVPSKYSLVILEAKRAHELEAGAPATQGFKSEKSTLRALEEIESGNVTIHPDPEGKREAVRRRIEEEKR RKEEEEKKIKEQIAKEKEDGEKIZ

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LASLITSIIMFYVGFDVLRDTIQKILSREETVIDPLGATLGIISAAIMFVVYLYNTRLSKKSNSNALKAAAKDNLSDAVTSL GTAIAILASSFNYPIVDKLVAIIITFFILKTAYDIFIESSFSLSDGFDDRLLEDYQKAIMEIPKISKVKSQRGRTYGSNIYLDIT LEMNPDLSVFESHEIADQVESMLEERFGVFDTDVHIEPAPIPEDEILDNVYKKLLMREQLIDQGNQLEELLTDDFVYIRQ DGEQMDKEAYKTKKELNSAIKDIQITSISQKTKLICYELDGIIHTSIWRRHETWQNIFHQETKKEZ

MTIKLVATDMDGTFLDGNGRFDMDRLKSLLVSYKEKGIYFAVASGRGFLSLEKLFAGVRDDIIFIAENGSLVEYQGQDL YEATMSRDFYLATFEKLKTSPYVDINKLLLTGKKGSYVLDTVDETYLKVSQHYNENIQKVASLEDITDDIFKFTTNFTEE TLEDGEAWVNENVPGVKAMTTGFESIDIVLDYVDKGVAIVELVKKLGITMDQVMAFGDNLNDLHMMQVVGHPVAPE NARPEILELAKTVIGHHKERSVIAYMEGLZ

MADIKLIALDLDGTLLTTDKRLTDRTKETLQAARDRGIKVVLTTGRPLKAMDFFLHELGTDGQEDEYTITFNGGLVQK NTGEILDKTVFSYDDVARLYEETEKLSLPLDAISEGTVYQIQSDQESLYAKFNPALTFVPVDFEDLSSQMTYNKCVTAFA QEPLDAAIQKISPELFDQYEIFKSREMLLEWSPKNVHKATGLAKLISHLGIDQSQVMACGDEANDLSMIEWAGLGVAM QNAVPEVKAAANVVTPMTNDEEAVAWAIEEYVLKENZ

MESLLILLLIANLAGLFLIWQRQDRQEKHLSKSLEDQADHLSDQLDYRFDQARQASQLDQKDLEVVVSDRLQEVRIELH QGLTQVRQEMTDNLLQTRDKTDQRLQALQESNEQRLEQMRQTVEEKLEKTLQTRLQASFETVSKQLESVNRGLGEMQ TVARDVGALNKVLSGTKTRGILGELQLGQIIEDIMTPAQYEREYATVENSSERVEYAIKLPGQGDQEYVYLPIDSKFPLA DYYRLEEAYETGDKDEIERCRKSLLASVKRFARDIRNKYIAPPRTTNFGVLFVPTEGLYSEIVRNPVFFDDLRREEQIIVA GPSTLSALLNSLSVGFKTLNIQKSADHISKTLASVKTEFGKFGGILVKAQKHLQHASGNIDELLNRRTIAIERTLRHIELSE GEPALDLLHFQENEEEYEDZ

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MSEKAKKGFKMPSSYTVLLIIIAIMAVLTWFIPAGAFIEGIYETQPQNPQGIWDVLMAPIRAMLGTHPEEGSLIKETSAAID VAFFILMVGGFLGIVNKTGALDVGIASIVKKYKGREKMLILVLMPLFALGGTTYGMGEETMAFYPLLVPVMMAVGFDS LTGVAIILLGSQIGCLASTLNPFATGIASATAGVGTGDGIVLRLIFWVTLTALSTWFVYRYADKIQKDPTKSLVYSTRKED LKHFNVEESSSVESTLSSKQKSVLFLFVLTFILMVLSFIPWTDLGVTIFDDFNTWLTGLPVIGNIVGSSTSALGTWYFPEG AMLFAFMGILIGVIYGLKEDKIISSFMNGAADLLSVALIVAIARGIQVIMNDGMITDTILNWGKEGLSGLSSQVFIVVTYIF YLPMSFLIPSSSGLASATMGIMAPLGEFVNVRPSLIITAYQSASGVLNLIAPTSGIVMGALALGRINIGTWWKFMGKLVVA IIVVTIALLLLGTFLPFLZ

MSNSFVKLLVSQLFANLADIFFRVTIIANIYIISKSVIATSLVPILIGISSFVASLLVPLVTKRLALNRVLSLSQFGKTILLAIL VGMFTVMQSVAPLVTYLFVVAISILDGFAAPVSYAIVPRYATDLGKANSALSMTGEAVQLIGWGLGGLLFATIGLLPTT CINLVLYIISSFLMLFLPNAEVEVLESETNLEILLKGWKLVARNPRLRLFVSANLLEIFSNTIWVSSIILVFVTELLNKTESY WGYSNTAYSIGIIISGLIAFRLSEKFLAAKWEPQLFTPNLKTIQNPCLSLDPGWFLFSPNGCFLLDKKEFPLYGISVEKNTK RKETHMNSLPNHHFQNKSFYQLSFDGGHLTQYGGLIFFQELFSQLKLKERISKYLVTNDQRRYCRYSDSDILVQFLFQLL

TGYGTDYACKELSADAYFPKLLEGGQLASQPTLSRFLSRTDEETVHSLRCLNLELVEFFLQFHQLNQLIVDIDSTHFTTYGKQEGVAYNAHYRAHGYHPLYAFEGKTGYCFNAQLRPGNRYCSEEADSFITPVLERFNQLLFRMDSGFATPKLYDLIEKTGQYYLIKLKKNTVLSRLGDLSLPCPQDEDLTILPHSAYSETLYQAGSWSHKRRVCQFSERKEGNLFYDVISLVTNMTSGTSQDQFQLYRGRGQAENFIKEMKEGFFGDKTDSSTLIKNEVRMMMSCIAYNLYLFLKHLAGGDFQTLTIKRFRHLFLHVVGKCVRTGRKQLLKLSSLYAYSELFSALYSRIRKVNLNLPVPYEPPRRKASLMMHZ

MMEFFQQLPHLEPYGNPQYFVYVIAATLPIFIGLFFKKRFAWYEVLVSLFFIVTMLVGGKTNQLAALGIYLCWEILLLLFYKHYRKSKDGKWVFYLVSFLSLLPIIFVKVQPAINGTQSLLGFLGISYLTFRSVGIVIELRDGVIKDFTLWEFLRFLLFMPTFSSGPIDRFKRFNENYQAIPERDELMDMLDESVRYIMWGFLYKFILAHVLGETLLPPLKNLALQSGGFFNLYALAVMYTFGLELFFDFAGYSMFALAISNLMGIRSPINFNKPFLSRDLKEFWNRWHMSLSFWFRDFVFMRMVMVLTRKKVFKNRNVTSSMAYIVNMLIMGFWHGVTWYYIAYGLFHGLGLVINDAWVRKKKTLNKERKKAGKAALPENRWIQLLGMVVTFHVVMLSFLIFSGFLNNLWFKKZ

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MIEYKNVALRYTEKDVLRDVNLQIEDGEFMVLVGPSGSGKTTMLKMINRLLEPTDGNIYMDGKRIKDYDERELRLSTGYVLQAIALFPNLTVAENIALIPEMKGWSKEEITKKTEELLAKVGLPVAEYGHRLPSELSGGEQQRVGIVRAMIGQPKIFLMDEPFSALDAISRKQLQVLTKELHKEFGMTTIFVTHDTDEALKLADRIAVLQDGEIRQVANPETILKAPATDFVADLFGGSVHDZ

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TZ

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MANKNTSTTRRRPSKAELERKEAIQRMLISLGIAILLIFAAFKLGAAGITLYNLIRLLVGSLAYLAIFGLLIYLFFFKWIRK
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ILILVGSLLVSPWSVYDIAEFFSRGFAKWWEGHERRKEERFVKQEEKARQKAEKEARLEQEETEKALLDLPPVDMETGE ILTEEAVQNLPPIPEEKWVEPEIILPQAELKFPEQEDDSDDEDVQVDFSAKEALEYKLPSLQLFAPDKPKDQSKEKKIVRE NIKILEATFASFGIKVTVERAEIGPSVTKYEVKPAVGVRVNRISNLSDDLALALAAKDVRIEAPIPGKSLIGIEVPNSDIATV SFRELWEQSQTKAENFLEIPLGKAVNGTARAFDLSKMPHLLVAGSTGSGKSVAVNGIIASILMKARPDQVKFMMVDPK MVELSVYNDIPHLLIPVVTNPRKASKALQKVVDEMENRYELFAKVGVRNIAGFNAKVEEFNSQSEYKQIPLPFIVVIVDE LADLMMVASKEVEDAIIRLGQKARAAGIHMILATQRPSVDVISGLIKANVPSRVAFAVSSGTDSRTILDENGAEKLLGRG DMLFKPIDENHPVRLQGSFISDDDVERIVNFIKTQADADYDESFDPGEVSENEGEFSDGDAGGDPLFEEAKSLVIETQKA SASMIQRRLSVGFNRATRLMEELEIAGVIGPAEGTKPRKVLQQZ

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MNKSEHRHQLIRALITKNKIHTQAELQALLAENDIQVTQATLSRDIKNMNLSKVREEDSAYYVLNNGSISKWEKRLELY MEDALVWMRPVQHQVLLKTLPGLAQSFGSIIDTLSFPDAIATLCGNDVCLIICEDADTAQKCFEELKKFAPPFFFEEZ

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MELFMKITNYEIYKLKKSGLTNQQILKVLEYGENVDQELLLGDIADISGCRNPAVFMERYFQIDDAHLSKEFQKFPSFSIL DDCYPWDLSEIYDAPVLLFYKGNLDLLKFPKVAVVGSRACSKQGAKSVEKVIQGLENELVIVSGLAKGIDTAAHMAAL QNGGKTIAVIGTGLDVFYPKANKRLQDYIGNDHLVLSEYGPGEQPLKFHFPARNRIIAGLCRGVIVAEAKMRSGSLITCE RAMEEGRDVFAIPGSILDGLSDGCHHLIQEGAKLVTSGQDVLAEFEFZ

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EZ

MELNTHNAEILLSAANKSHYPQDELPEIALAGRSNVGKSSFINTMLNRKNLARTSGKPGKTQLLNFFNIDDKMRFVDVP
GYGYARVSKKEREKWGCMIEEYLTTRENLRAVVSLVDLRHDPSADDVQMYEFLKYYEIPVIIVATKADKIPRGKWNKH
ESAIKKKLNFDPSDDFILFSSVSKAGMDEAWDAILEKLZ

MTKKQLHLVIVTGMSGAGKTVAIQSFEDLGYFTIDNMPPALLPKFLQLVEIKEDNPKLALVVDMRSRSFFSEIQAVLDEL
ENQDGLDFKILFLDAADKELVARYKETRRSHPLAADGRILDGIKLERELLAPLKNMSQNVVDTTELTPRELRKTLAEQF
SDQEQAQSFRIEVMSFGFKYGIPIDADLVFDVRFLPNPYYLPELRNQTGVDEPVYDYVMNHPESEDFYQHLLALIEPILP
SYQKEGKSVLTIAMGCTGGQHRSVAFAKRLAQDLSKNWSVNEGHRDKDRRKETVNRSZ

MRKPKITVIGGGTGSPVILKSLREKDVEIAAIVTVADDGGSSGELRKNMQQLTPPGDLRNVLVAMSDMPKFYEKVFQYR
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VYVTNALNDDTPLASRRVVQTILESDMIVLGPGSLFTSILPNIVIKEIGRALLETKAEIAYVCNIMTQRGETEHFTDSDHV
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I
IQVKKZ

MKNLIKLLIIRLIVNLADSVFYIVALWHVSNNYSSSMFLGIFIAVNYLPDLLLIFFGPVIDRVNPQKILIISILVQLAVAVIFL
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LALFILLLLKFRTSNANIENFSFKYYKREVLQGTKFILNNKLLFKTSISLTLINFFYSFQTVVVPIFSIRYFDGPIFYGIFLTIA
GLGGILGNMLAPIVIKYLKSNQIVGVFLFLNGSSWLVAIVIKDYTLSLILFFVCFMSKGVFNIIFNSLYQQIPPHQLLGRVN
TTIDSIISFGMPIGSLVAGTLIDLNIELVLIAISIPYFLFSYIFYTDNGLKEFSIYZ

MMSNKNKEILIFAILYTVLFMFDGVKLLASLMPSAIANYLVYVVLALYGSFLFKDRLIQQWKEIRKTKRKFFFGVLTGW
LFLILMTVVFEFVSEMLKQFVGLDGQGLNQSNIQSTFQEQPLLIAVFACVIGPLVEELFFRQVLLHYLQERLSGLLSIILV
GLVFALTHMHSLALSEWIGAVGYLGGGLAFSIIYVKEKENIYYPLLVHMLSNSLSLIILAISIVKZ

LKKPIIEFKNVSKVFEDSNTKVLKDINFELEEGKFYTLLGASGSGKSTILNIIAGLLDATTGDIMLDGVRINDIPTNKRDVHTVFQSYALFPHMNVFENVAFPLRLRKIDKKEIEQRVAEVLKMVQLEGYEKRSIRKLSGGQRQRVAIARAIINQPRVVLLDEPLSALDLKLRTDMQYELRELQQRLGITFVFVTHDQEEALAMSDWIFVMNDGEIVQSGTPVDIYDEPINHFVATFIGESNILPGTMIEDYLVEFNGKRFEAVDGGMKPNEPVEVVIRPEDLRITLPEEGKLQVKVDTQLFRGVHYEIIAYDELGNEWMIHSTRKAIVGEEIGLDFEPEDIHIMRLNETEEEFDARIEEYVEIEEQEAGLINAIEEERDEENKLZ

MKSMRILFLLALIQISLSSCFLWKECILSFKQSTAFFIGSMVFVSGICAGVNYLYTRKQEVHSVLASKKSVKLFYSMLLLINLLGAVLVLSDNLFIKNTLQQELVDFLLPSFFFLFGLDLLIFLPLKKYVRDFLAMLDRKKTVLVTILATLLFLRNPMTIVSLLIYIGLGLFFAAYLVPNSVKKEVSFYGHIFRDLVLVIVTLIFFZ

MVKKIIGMVLALLSVTVVGVGVFAYTIYQQGTETLAKTYKKIGEETKVIEATEPLTILLMGVDTGNVERTETWVGRSDSMILMTVNPKTKKTTMMSLERDILTRIESGNGQAHEAKLNSAYADGGAELAIETIQKMMNIHIDRYVMVNMRGLQKLVDAVGGITVNNILGFPISISDQEEFNTISIGVGEQHIGGEEALVYARMRYQDPEGDYGRQKRQREVIQKVMEKALSLNSIGHYQEILKALSDNMQTNIDLSAKSIPNLLGYKDSFKTIETQQLQGEGEILQGVSYQIVSRAHMLEMQNLLRRSLGQEEVTQLETNAVLFEDLFGRAPVGDEDNZ

MKKQAYVIIALTSFLFVFFFSHSLLEILDFDWSIFLHDVEKTEKFVFLLLVFSMSMTCLLALFWRGIEELSLRKMQANLKRLLAGQEVVQVADPDLDASFKSLSGKLNLLTEALQKAENQSLAQEEEIIEKERKRIARDLHDTVSQELFAAHMILSGISQQALKLDREKMQTQLQSVTAILETAQKDLRVLLLHLRPVELEQKSLIEGIQILLKELEDKSDLRVSLKQNMTKLPKKIEEHIFRILQELISNTLRHAQASCLDVYLYQTDVELQLKVVDNGIGFQLGSLDDLSYGLRNIKERVEDMAGTVQLLTAPKQGLAVDIRIPLLDKEZ

MIVSIISQGFVWAILGLGIFMTFRILNFPDMTTEGSFPLGGAVAVTLITKGVNPFLATLVAVGAGCLAGMAAGLLYTKGKIPTLLSGILVMTSCHSIMLLIMGRANLGLLGTKQIQDVLPFDSDLNQLLTGLIFVSIVIALMLFFLDTKLGQAYIATGDNPDMARSFGIHTGRMELMGLVLSNGVIALAGALIAQQEGYADVSRGIGVIVVGLASLIIGEVIFKSLSLAERLVTIVVGSIAYQFLVWAVIALGFNTSYLRLYSALILAVCLMIPTFKQTILKGAKLSKZ

MKKMKVWSTVLATGVALTTLAACSGGSNSTTASSSEEKADKSQELVIYSNSVSNGRGDWLTAKAKEAGFNIKMVDIAGAQLADRVIAEKNNAVADMVFGIGAVDSNKIRDQKLLVQYKPKWLDKIDQSLSDKDNYYNPVIVQPLVLIGAPDVKEMPKDWTELGSKYKGKYSISGLQGGTGRAILASILVRYLDDKGELGVSEKGWEVAKEYLKNAYTLQKGESSIVKMLDKEDPIQYGMMWGSGALVGQKEQNVVFKVMTPEIGVPFVTEQTMVLSTSKKQALAKEFIDWFGQSEIQVEYSKNFGSIPANKDALKDLPEDTKKFVDQVKPQNIDWEAVGKHLDEWVEKAELEYVQZ

MIKFDNIQIKYGDFVAIDNLNLDIHEGEFFTFLGPSGCGKSTTLRALVGFLDPSSGSIEVNGTDVTHLEPEKRGIGIVFQSYALFPTMTVFDNIAFGLKVKKVAPDVIKAKVSAVAAKIKISDQQLQRNVSELSGGQQQRVALARALVLEPKILCLDEPLSNLDAKLRVDLRKELKRLQKELGITTLYVTHDQEEALTLSDRIAVFNNGYIEQVGTPVEIYHNSQTEFVCDFIGDINVLTDETVHEVLLKNTSVFLEDKKGYIRLEKVRFNRETEQDFILKGTIIDVEFSGVTIHYTIKVSESQILNVTSIDSQAAIRSVGESVELFITPSDVLQFZ

MRHKLNLKDWLIRLGLIWFLVTFIIYPNFDLVVNVFVKGGEFSLDAVHRVLKSQRALQSIMNSFKLAFSLIITVNVVGILCVLFTEYFDIKGAKILKLGYMTSLIYGGVVLATGYKFVYGPYGLITKFLQNVIPSLDPNWFIGYGAVLFIMTFSGTANHTLFLTNTIRSVDYHTIEAARNMGAKPFTVFRKVVLPTLIPTLFALTIMVFLSGLSAVAAPMIVGGKEFQTINPMIITFAGMGNSRDLAALLAIILGIATTILLTIMNKIEKGGNYISISKTKAPLKKQKIASKPWNIIAHIVAYGLFTVFMLPLIFIVLYSFTDPVAIQTGNLTLSNFTLENYRLFFSNSAAFSPFLVSFIYSIIAATTATILAVVFARVVRKHKSRFDFLFEYGALLPWLLPSTLLAVSLLFTFNQPQFLVLNQILVGSLVILLIAYIVVKIPFSYRMVRAILFSVDDEMEDAARSMGASPFYTMMKVIIPFILPVVLSVIALNFNSLLTDFDLSVFLYHPLAQPLGITIRSAGDETATSNAQALVFVYTIVLMIISGTVLYFTQRPGRKVRKZ

Table 3

<u>**ID201 - 4106.4**</u>

ATGATAAAAAATCCTAAATTATTAACCAAGTCTTTTTTAAGAAGTTTTGCAATTCTAGGTGGTGTTGGTCTAGTCAT
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GCTATCAAATTATATCGTGATGTTAGATATGTCTGTTAGTACAGCAGATGGTAAACAGGTAACCGTGCAATTTGTTC
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CAAAAGGTTTCCTTTGTCCGCGGAGCATCACATGAGTTGAAAACCCCTTTAGCCAGTCTTAGAATTATCCTAGAGAA
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GCCACTTATTAGAAGAAGTACTGGAGTCTTCTAAATTCCAAGAGTGGACAGAGTGTCGTGAGACCTTGACTGTTAAG
CCAGTTTTAGTAGATATTTTATCACGTTATCAAGAATTAGCTCATTCAATAGGTGTTACAATTGAAAATCAATTGAC
AGATGCTACCAGGGTCGTCATGAGTCTTAGGGCATTGGATAAGGTTTTGACAAACCTGATTAGTAATGCAATTAAAT
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AGTGACCAAGAACTAGAACATTTATTTGATATATTCTATCATTCTCAAATCGTGACAGATAAGGATGAAAGTTCCGG
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TGGAATTTAAGATTAGCTTGTAG

MIKNPKLLTKSFLRSFAILGGVGLVIHIAIYLTFPFYYIQLEGEKFNESARVFTEYLKTKTSDEIPSLLQSYSKSLT
ISAHLKRDIVDKRLPLVHDLDIKDGKLSNYIVMLDMSVSTADGKQVTVQFVHGVDVYKEAKNILLLYLPYTFLVTIA
FSFVFSYFYTKRLLNPLFYISEVTSKMQDLDDNIRFDESRKDEVGEVGKQINGMYEHLLKVIYELESRNEQIVKLQN
QKVSFVRGASHELKTPLASLRIILENMQHNIGDYKDHPKYIAKSINKIDQMSHLLEEVLESSKFQEWTECRETLTVK
PVLVDILSRYQELAHSIGVTIENQLTDATRVVMSLRALDKVLTNLISNAIKYSDKNGRVIISEQDGYLSIKNTCAPL
SDQELEHLFDIFYHSQIVTDKDESSGLGLYIVNNILESYQMDYSFLPYEHGMEFKISLZ

<u>**ID202 - 4106.9**</u>

ATGGATAAAATTATTAAAACTATATCAGAAAGCGGAGCCTTTCGTGCTTTTGTCCTTGATAGCACTGAAACCGTCCG
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AGTCCTAGTCGGACCTTTTGTTGGAAATGGTCAATTCCTCGTTATCACAGACTACGGTACTGGAAATCCTTACAACT
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GCGACGACCATATCGAAGCCCTCCTCAAGGCTATCTACGGGGACGAAGCCTACAAGCGTCTTTCTGAAGAAGAAATC
CGTTTCCAATGTGACTGTAGCCATGAACGCTTTATGAACGCTCTTGCCAGCCTTCCAAGCTCAGACTTACAGGAAAT
GAAAGAGGAAGACCACGGGGCAGAAATCACTTGTCAATTCTGCCAAACTACTTACAACTTTGATGAAAAGGACCTGG
AGGAACTCATTCGTGACAAATCTTAA

MDKIIKTISESGAFRAFVLDSTETVRTAQEKHQTQASSTVALGRTLIASQILAANEKGNTKLTVKVLGSSSLGAIIT
VADTKGNVKGYVQNPGVDIKKTATGEVLVGPFVGNGQFLVITDYGTGNPYNSITPLISGEIGEDLAFYLTESQQTPS
AVGLNVLLDEEDKVKVAGGFLVQVLPGAKKEEIARFEKRIQEMPAISTLLESDDHIEALLKAIYGDEAYKRLSEEEI
RFQCDCSHERFMNALASLPSSDLQEMKEEDHGAEITCQFCQTTYNFDEKDLEELIRDKSZ

<u>**ID203 - 4115**</u>

ATGAAATCAATAACTAAAAAGATTAAAGCAACTCTTGCAGGAGTAGCTGCCTTGTTTGCAGTATTTGCTCCATCATT
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CAACAGTTGAAAAATTGGCAGAAAACAACCACATTGATAACATTCATTTGATTTATGTTGATCAAGAGTTGGTTATC
GATGGCCCTGTAGCGCCTGTTGCAACACCAGCGCCAGCTACTTATGCGGCACCAGCCGCTCAAGATGAAACTGTTTC

AGCTCCAGTAGCAGAAACTCCAGTAGTAAGTGAAACAGTTGTTTCAACTGTAAGCGGATCTGAAGCAGAAGCCAAAG
AATGGATCGCTCAAAAAGAATCAGGTGGTAGTATACAGCTACAAATGGACGTTATATCGGACGTTACCAATTAA

MKSITKKIKATLAGVAALFAVFAPSFVSAQESSTYTVKEGDTLSEIAETHNTTVEKLAENNHIDNIHLIYVDQELVI
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ID204 - 4117.1

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GACAGCCAATGAATGGATTTGGGATAAGGAATCTTGGTTTTACCTCAAATCTGATGGGAAAATAGCTGAAAAAGAAT
GGGTCTACGATTCTCATAGTCAAGCTTGGTACTACTTCAAATCTGGTGGCTACATGGCGAAAAATGAGACAGTAGAT
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AAATCAATGAGAAGCTAGGTGGCAAAGATTAG

MNLGEFWYNKINKNRGRRLMKKVRFIFLALLFFLASPEGAMASDGTWQGKQYLKEDGSQAANEWVFDTHYQSWFYIK
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WFYIKADGQHAEKEWLQIKGKDYYFKSGGYLLTSQWINQAYVNASGAKVQQGWLFDKQYQSWFYIKENGNYADKEWI
FENGHYYYLKSGGYMAANEWIWDKESWFYLKFDGKMAEKEWVYDSHSQAWYYFKSGGYMTANEWIWDKESWFYLKSD
GKIAEKEWVYDSHSQAWYYFKSGGYMTANEWIWDKESWFYLKSDGKIAEKEWVYDSHSQAWYYFKSGGYMAKNETVD
GYQLGSDGKWLGGKTTNENAAYYQVVPVTANVYDSDGEKLSYISQGSVVWLDKDRKSDDKRLAITISGLSGYMKTED
LQALDASKDFIPYYESDGHRFYHYVAQNASIPVASHLSDMEVGKKYYSADGLHFDGFKLENPFLFKDLTEATNYSAE
ELDKVFSLLNINNSLLENKGATFKEAEEHYHINALYLLAHSALESNWGRSKIAKDKNNFFGITAYDTTPYLSAKTFD
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ID205 - 4118.1

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AKQLSAKDPNNKEFYEKNLKEYTDKLDKLDKESKDKFNKIPAEKKLIVTSEGAFKYFSKAYGVPSAYIWEINTEEEG
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KZ

<u>ID206 - 4119.1</u>

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MEWYKKIGLLATTGLALFGLGACSNYGKSADGTVTIEYFNQKKEMTKTLEEITRDFEKENPKIKVKVVNVPNAGEVL
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ELGLKVPETWDEFEQLVKDIVAKGQTPFGIAGADAWTLNGYNQLAFATATGGKEANQYLRYSQPNAIKLSDPIMKD
DIKVMDILRINGSKQKNWEGAGYTDVIGAFARGDVLMTPNGSWAITAINEQKPNFKIGTFMIPGKEKGQSLTVGAGD
LAWSISATTKHPKEANAFVEYMTRPEVMQKYYDVDGSPTAIEGVKQAGEDSPLAGMTEYAFTDRHLVWLQQYWTSEA
DFHTLTMNYVLTGDKQGMVNDLNAFFNPMKADVDZ

<u>ID207 - 4123.1</u>

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GTCAGTTGGATGCGCGTGAGTGGGTTCGTGTGCTCAAGGAAACGGGCTTCAAAAAGTTGATTTTGGTGGTCAAGCAC
CACGATGGCTTTGTCCTTTATCCGACAGCTCACACAGATTATTCGGTTAAGGTCAGTCCTTGGAGGAGAGGAAAGGG
CGACTTGCTCCTTGAAGTATCCCAAGCTGCCACAGAGTTTGATATGGATATGGGGGTCTACCTGTCACCGTGGGATG
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GCACCAGTATCCGCTGGATTGGCAATGAACGAGGGTATGCAGGTGATCCACTGTGGCAAAAGGTGAATCCTGATAAA
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TTCACTCAGTAGGGCGAGGAACTCCACTCTTGCTTAATATTCCGCCGAATCAAGCTGGGCTCTTTGATGCAAAGGAT
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ID208 - 4125.12

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ID209 - 4126.3

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ID210 - 4127.1

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ID211 - 4127.2

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ID212 - 4136.1

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ID213 - 4137.3

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VER

ID214 - 4185

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ID215 - 4211.1

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VQIENVMVSDKEVSITSDQETLDRIDKIIAVLPTSERITGNYSGSVPLQAIDRNGVVLPAVITPFDTIMKVTTKPVA
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<u>ID216 - 4127.3</u>

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Table 4

<u>ID301</u>

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<u>ID302</u>

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ID303

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ID304

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ID305

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ID306

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ID307

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ID308

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ID309

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ID310

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ID311

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LVKEAVRKVGDGYVFEENGVSRYIPAKDLSAETAAGIDSKLAKQESLSHKLGAKKTDLPSSDREFYNKAYDLLARIH
QDLLDNKGRQVDFEALDNLLERLKDVPSDKVKLVDDILAFLAPIRHPERLGKPNAQITYTDDEIQVAKLAGKYTTED
GYIFDPRDITSDEGDAYVTPHMTHSHWIKKDSLSEAERAAAQAYAKEKGLTPPSTDHQDSGNTEAKGAEAIYNRVKA
AKKVPLDRMPYNLQYTVEVKNGSLIIPHYDHYHNIKFEWFDEGLYEAPKGYTLEDLLATVKYYVEHPNERPHSDNGF
GNASDHVQRNKNGQADTNQTEKPSEEKPQTEKPEEETPREEKPQSEKPESPKPTEEPEESPEESEEPQVETEKVEEK
LREAEDLLGKIQDPIIKSNAKETLTGLKNNLLFGTQDNNTIMAEAEKLLALLKESKZ

<u>ID312</u>

ATGGAGGGATTGGTTAGAGTGCATTTATTGCCTGTATTTGGCGATTACAAGCTATCTAAACTTACTACGCCTATTCT
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GTTCCACGCAAACAGCAAAAAGAAAAGGCTGCTGTCAAATACTTAGACAACAAAGAATTAAAACAGTTTCTTGATTA
TTTAGATGCTCTGGATCAATCAAATTATGAGAACTTATTTGATGTTGTTCTGTATAAGACTTTATTGGCCACTGGTT
GCCGTATTAGTGAGGCTCTGGCTCTTGAATGGTCTGATATTGACCTAGAAAGCGGTGTTATCAGCATCAATAAGACA
CTAAACCGCTATCAGGAAATAAACTCACCTAAATCAAGCGCTGGTTATCGTGATATACCAATAGACAAAGCCACATT
ACTTTTACTGAAACAATACAAAAACCGTCAACAAATTCAGTCTTGGAAATTAGGCCGATCTGAAACAGTTGTATTCT
CTGTATTTACGGAGAAATATGCTTATGCTTGTAACTTACGCAAACGCCTAAATAAGCATTTTGATGCTGCTGGAGTA
ACTAACGTATCATTTCATGGTTTCCGCCATACACATACTACTATGATGCTCTATGCTCAGGTTAGCCCGAAAGATGT
TCAGTATAGATTAGGCCACTCTAATTTAATGATCACTGAAAATACTTACTGGCATACTAACCAAGAGAATGCAAAAA
AAGCCGTCTCAAATTATGAAACAGCTATCAACAATTTATAA

MEGLVRVHLLPVFGDYKLSKLTTPILQQQVNKWADKANKGEKGAFANYSLLHNMNKRILKYGVAIQVIQYNPANDVI
VPRKQQKEKAAVKYLDNKELKQFLDYLDALDQSNYENLFDVVLYKTLLATGCRISEALALEWSDIDLESGVISINKT
LNRYQEINSPKSSAGYRDIPIDKATLLLLKQYKNRQQIQSWKLGRSETVVFSVFTEKYAYACNLRKRLNKHFDAAGV
TNVSFHGFRHTHTTMMLYAQVSPKDVQYRLGHSNLMITENTYWHTNQENAKKAVSNYETAINNLZ

CLAIMS:

1. A *Streptococcus pneumoniae* protein or polypeptide having a sequence selected from those shown in table 2.

2. A *Streptococcus pneumoniae* protein or polypeptide having a sequence selected from those shown in table 4.

3. A protein or polypeptide as claimed in claim 1 or claim 2 provided in substantially pure form.

4. A protein or polypeptide which is substantially identical to one defined in any one of claims 1 to 3.

5. A homologue or derivative of a protein or polypeptide as defined in any one of claims 1 to 4.

6. An antigenic and/or immunogenic fragment of a protein or polypeptide as defined
in Tables 2-4.

7. A nucleic acid molecule comprising or consisting of a sequence which is:

(i) any of the DNA sequences set out in Table 1 or their RNA equivalents;

(ii) a sequence which is complementary to any of the sequences of (i);

(iii) a sequence which codes for the same protein or polypeptide, as those sequences of (i) or (ii);

(iv) a sequence which is substantially identical with any of those of (i), (ii) and (iii);

(v) a sequence which codes for a homologue, derivative or fragment of a protein as defined in Table 1.

8. A nucleic acid molecule comprising or consisting of a sequence which is:

(i) any of the DNA sequences set out in Table 4 or their RNA equivalents;

(ii) a sequence which is complementary to any of the sequences of (i);

(iii) a sequence which codes for the same protein or polypeptide, as those sequences of (i) or (ii);

(iv) a sequence which is substantially identical with any of those of (i), (ii) and (iii);

(v) a sequence which codes for a homologue, derivative or fragment of a protein as defined in Table 4.

9. The use of a protein or polypeptide having a sequence selected from those shown in Tables 2-4, or homologues, derivatives and/or fragments thereof, as an immunogen and/or antigen.

10. An immunogenic and/or antigenic composition comprising one or more proteins or polypeptides selected from those whose sequences are shown in Tables 2-

4, or homologues or derivatives thereof, and/or fragments of any of these.

11. An immunogenic and/or antigenic composition as claimed in claim 10 which is a vaccine or is for use in a diagnostic assay.

12. A vaccine as claimed in claim 11 which comprises one or more additional components selected from excipients, diluents, adjuvants or the like.

13. A vaccine composition comprising one or more nucleic acid sequences as defined in Tables 1, 3 or 4.

14. A method for the detection/diagnosis of *S.pneumoniae* which comprises the step of bringing into contact a sample to be tested with at least one protein or polypeptide as defined in Tables 2-4, or homologue, derivative or fragment thereof.

15. An antibody capable of binding to a protein or polypeptide as defined in Tables 2-4, or for a homologue, derivative or fragment thereof.

16. An antibody as defined in claim 15 which is a monoclonal antibody.

17. A method for the detection/diagnosis of *S.pneumoniae* which comprises the step of bringing into contact a sample to be tested and at least one antibody as define din claim 15 or claim 16.

18. A method for the detection/diagnosis of *S.pneumoniae* which comprises the step of bringing into contact a sample to be tested with at least one nucleic acid sequence as defined in claim 7 or claim 8.

19. A method of determining whether a protein or polypeptide as defined in Tables 2-4 represents a potential anti-microbial target which comprises inactivating said protein or polypeptide and determining whether *S.pneumoniae* is still viable *in vitro* or *in vivo*.

20. The use of an agent capable of antagonising, inhibiting or otherwise interfering with the function or expression of a protein or polypeptide as defined in Tables 2-4 in the manufacture of a medicament for use in the treatment or prophylaxis of *S.pneumoniae* infection

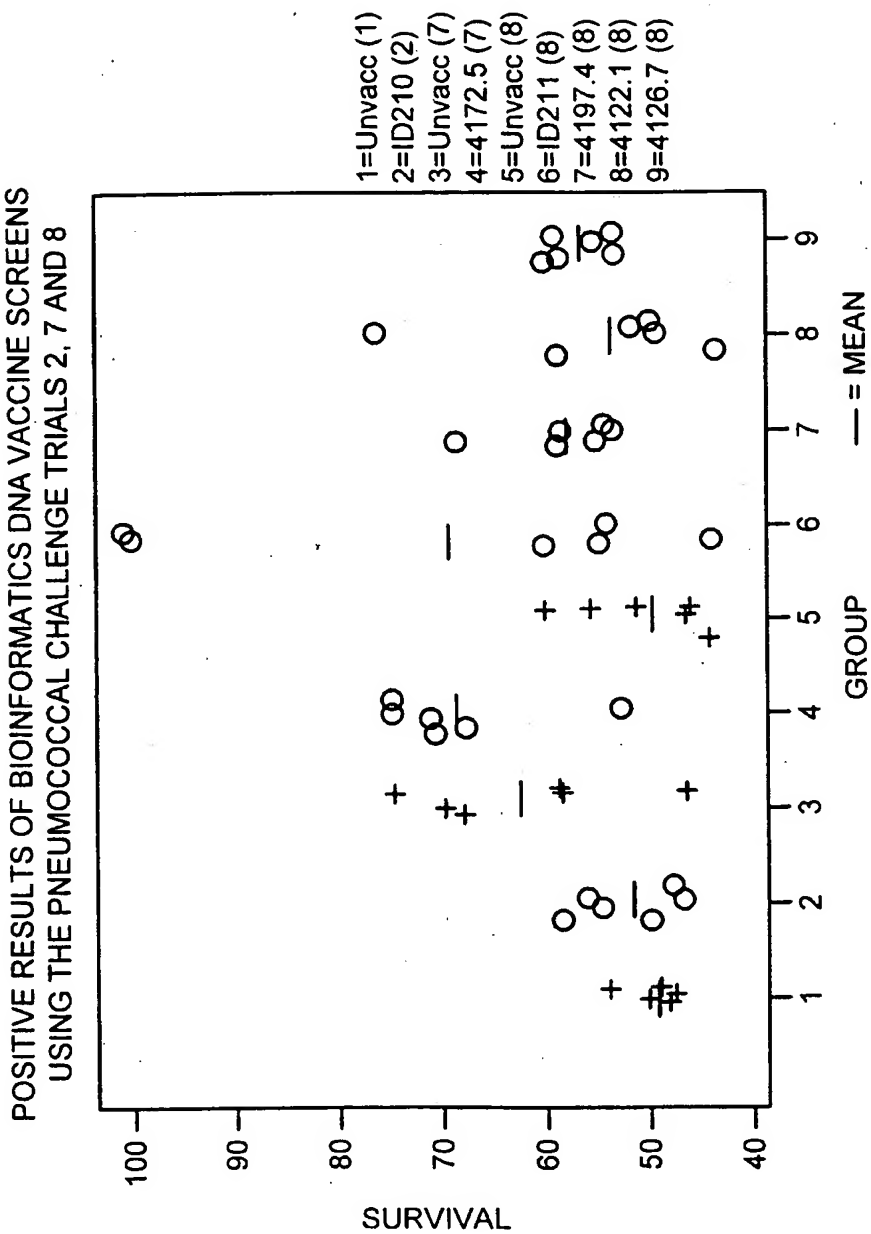
POSITIVE RESULTS OF BIOINFORMATICS DNA VACCINE SCREENS
USING THE PNEUMOCOCCAL CHALLENGE TRIALS 2, 7 AND 8
1=Unvacc (1)
2=ID210 (2)
3=Unvacc (7)
4=4172.5 (7)
5=Unvacc (8)
6=ID211 (8)
7=4197.4 (8)
8=4122.1 (8)
9=4126.7 (8)
SURVIVAL
100
90
80
70
60
50
40
GROUP
1
2
3
4
5
6
7
8
9
— = MEAN

FIG. 1

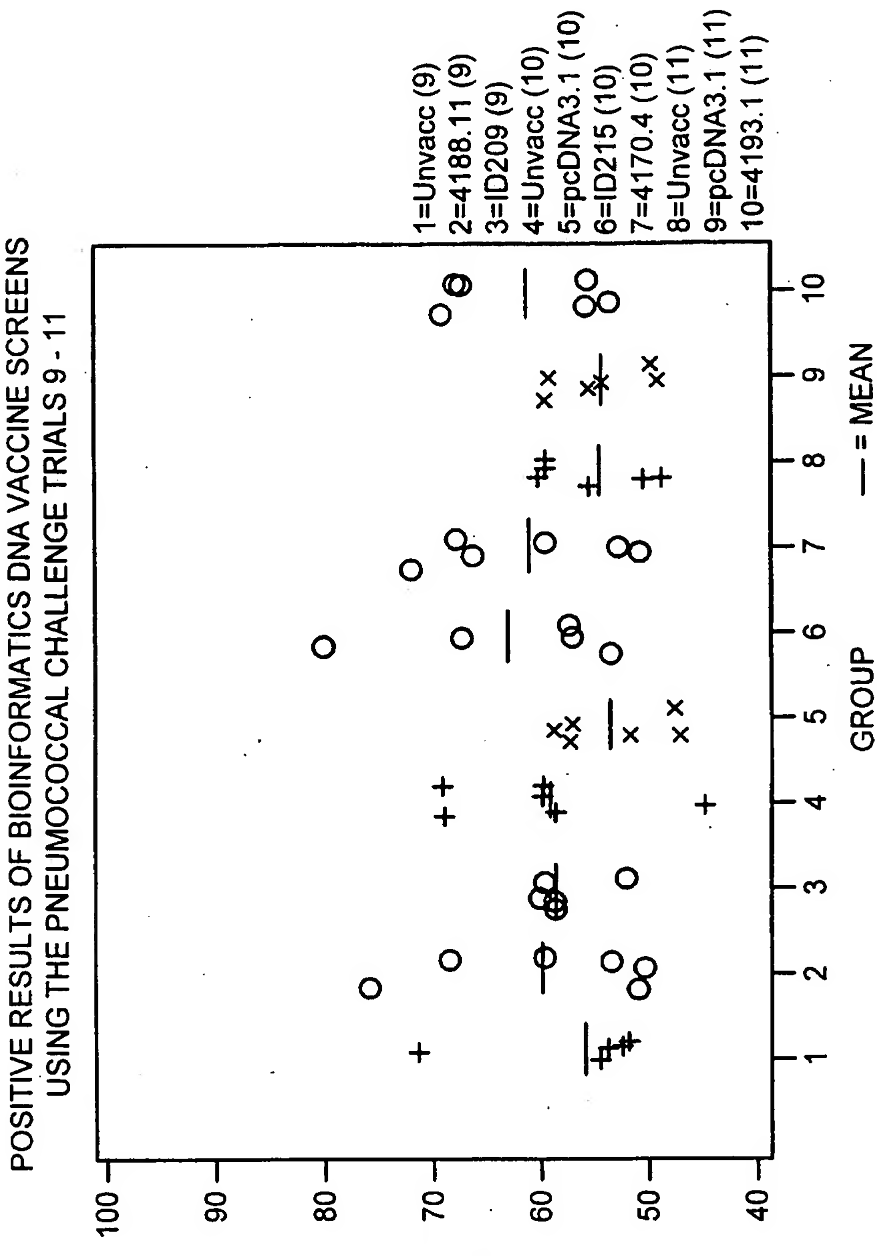
POSITIVE RESULTS OF BIOINFORMATICS DNA VACCINE SCREENS
USING THE PNEUMOCOCCAL CHALLENGE TRIALS 9 - 11
1=Unvacc (9)
2=4188.11 (9)
3=ID209 (9)
4=Unvacc (10)
5=pcDNA3.1 (10)
6=ID215 (10)
7=4170.4 (10)
8=Unvacc (11)
9=pcDNA3.1 (11)
10=4193.1 (11)
100
90
80
70
60
50
40
1
2
3
4
5
6
7
8
9
10
GROUP
— = MEAN

FIG. 2

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : C12N 15/31, C07K 14/315, 16/12, G01N 33/50, A61K 39/09, C12Q 1/68

A3

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60/125,164	19 March 1999 (19.03.99)	US

(71) Applicant *(for all designated States except US)*: MICROBIAL TECHNICS LIMITED [GB/GB]; 20 Trumpington Street, Cambridge CB2 1QA (GB).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: GILBERT, Christophe, François, Guy [FR/GB]; University of Cambridge, Dept. of Pathology, Tennis Court Road, Cambridge CB1 1PQ (GB). HANSBRO, Philip, Michael [GB/GB]; University of Cambridge, Dept. of Pathology, Tennis Court Road, Cambridge CB2 1QP (GB).

(74) Agents: CHAPMAN, Paul, William et al.; Kilburn & Strode, 20 Red Lion Street, London WC1R 4PJ (GB).

(81) Designated States: CN, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published

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(54) Title: STREPTOCOCCUS PNEUMONIAE PROTEINS AND NUCLEIC ACID MOLECULES

(57) Abstract

Novel protein antigens from *Streptococcus pneumoniae* are disclosed, together with nucleic acid sequences encoding them. Their use in vaccines and in screening methods is also described.

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INTERNATIONAL SEARCH REPORT

Inter   onal Application No
PCT/GB 99/02451

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/31 C07K14/315 C07K16/12 G01N33/50 A61K39/09
C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K G01N A61K C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 18931 A (DOUGHERTY BRIAN A ;HUMAN GENOME SCIENCES INC (US); ROSEN CRAIG A ()) 7 May 1998 (1998-05-07) SEQ ID NO 3,5,21,69,127,139,187 ---	1,3-7, 9-19
T	LANGE ROLAND ET AL: "Domain organization and molecular characterization of 13 two-component systems identified by genome sequencing of Streptococcus pneumoniae." GENE (AMSTERDAM) SEPT. 3, 1999, vol. 237, no. 1, pages 223-234, XP004183515 ISSN: 0378-1119 page 229; figures 1,3 ---	1,3-7

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 April 2000	09. 05. 2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Espen, J

3

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GUENZI ERIC ET AL: "A two-component signal-transducing system is involved in competence and penicillin susceptibility in laboratory mutants of Streptococcus pneumoniae." MOLECULAR MICROBIOLOGY 1994, vol. 12, no. 3, 1994, pages 505-515, XP000905352 ISSN: 0950-382X	
P,X	EP 0 885 966 A (SMITHKLINE BEECHAM PLC ;SMITHKLINE BEECHAM CORP (US)) 23 December 1998 (1998-12-23) SEQ ID NO 1,2,3	1,3-7, 9-19
P,X	EP 0 891 984 A (SMITHKLINE BEECHAM PLC ;SMITHKLINE BEECHAM CORP (US)) 20 January 1999 (1999-01-20) SEQ ID No 1,3,4	1,3-7, 9-19
X	WO 98 18930 A (HUMAN GENOME SCIENCES INC ;CHOI GIL H (US); HROMOCKYJ ALEX (US); J) 7 May 1998 (1998-05-07) SEQ ID NO 7,8,113,114,133,134,163,164,169,170	1,3-7, 9-19
P,X	FONTAN PA ET AL: "Streptococcus pmeumoniae choline transporter" EMBL DATABASE ENTRY AF162656, ACCESSION NUMBER AF162656,26 July 1999 (1999-07-26), XP002136498 nucleotide sequence and deduced amino acid sequence	1,3-7
X	FONTAN P A ET AL: "A choline transporter as a virulence determinant of Streptococcus pneumoniae." 97TH GENERAL MEETING OF THE AMERICAN SOCIETY FOR MICROBIOLOGY;MIAMI BEACH, FLORIDA, USA; MAY 4-8, 1997, vol. 97, 1997, page 103 XP000892162 Abstracts of the General Meeting of the American Society for Microbiology 1997 ISSN: 1060-2011 abstract	1,3-7
Y	TAKEMOTO K ET AL: "Putative ferric transport ATP-binding protein AFUC" SWISSPROT DATABASE ENTRY AFUC_ECOLI, ACCESSION NUMBER P37009, 1 June 1994 (1994-06-01), XP002136499 sequence	6,9

-/--

3

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	FLEISCHMANN RD ET AL: "Putative ferric transport ATP-binding protein AFUC" SWISSPROT DATABASE ENTRY AFUC_HAEIN, ACCESSION NUMBER P44531, 1 November 1995 (1995-11-01), XP002136500 sequence ---	6,9
Y	BLATTNER FR ET AL: "Spermidine/putrescine transport ATP-binding protein POTA" SWISSPROT DATABASE ENTRY POTA_ECOLI, ACCESSION NUMBER P23858, 1 November 1991 (1991-11-01), XP002136501 sequence ---	6,9
P,A	POLISSI ALESSANDRA ET AL: "Large-scale identification of virulence genes from Streptococcus pneumoniae." INFECTION AND IMMUNITY DEC., 1998, vol. 66, no. 12, December 1998 (1998-12), pages 5620-5629, XP002136502 ISSN: 0019-9567 ---	
A	DINTILHAC A ET AL: "The adc locus, which affects competence for genetic transformation in Streptococcus pneumoniae, encodes an ABC transporter with a putative lipoprotein homologous to a family of streptococcal adhesins." RESEARCH IN MICROBIOLOGY 1997, vol. 148, no. 2, 1997, pages 119-131, XP002115703 ISSN: 0923-2508 ---	
A	WO 95 06732 A (MASURE H ROBERT ;TUOMANEN ELAINE (US); PEARCE BARBARA J (US); UNIV) 9 March 1995 (1995-03-09) ---	
A	EP 0 622 081 A (UAB RESEARCH FOUNDATION) 2 November 1994 (1994-11-02) -----	

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INTERNATIONAL SEARCH REPORT

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [ ] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. [X] Claims Nos.: 20
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [X] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
1,3-7,9-19 (SEQ ID NO: 1,208; 27,235; 80,292; 132,344; 137,349; 162,178; 166,182)

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[X] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 20

Claim 20 relates to the use of an agent capable of antagonising, inhibiting or otherwise interfering with the function or expression of a protein or polypeptide as defined in Tables 2-4 in the manufacture of a medicament. Neither a true technical characterization is given for such an agent, nor is such an agent defined in the application. In consequence, the scope of said claim is ambiguous and vague, and its subject-matter is not suffieciently disclosed and supported (Art. 5 and 6 PCT).
No search can be carried out for such purely speculative claims whose wording is, in fact, a mere recitation of the result to be achieved.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: in part: 1,3-7,9-19; all as far as applicable

   Streptococcus (S.) pneumoniae protein or polypeptide having a sequence relating to SEQ ID No 1, antigenic and/or immunogenic fragment thereof; nucleic acid molecule relating to SEQ ID No 208, and vaccine comprising said nucleic acid; use of said protein or polypeptide as an immunogen and/or antigen; immunogenic and/or antigenic composition comprising said protein or polypeptide, and its use as a vaccine; antibody directed to said protein or polypeptide; method for the detection/diagnosis using either said protein/polypeptide, or said antibody, or said nucleic acid molecule; method of determining whether said protein or polypeptide represents a potential anti-microbial target

2-179. Claims: in part: 1-19; all as far as applicable

as invention 1 but limited to subject-matter relating SEQ ID Nos 2-151 (table 2), SEQ ID Nos 152-167 (table 3), and SEQ ID Nos 184-195 (table 4) and the corresponding nucleic acid molecules; wherein
invention 2 is limited to SEQ ID No 2,
invention 3 is limited to SEQ ID No 3, etc...
invention 179 is limited to SEQ ID No 195.

Patent document cited in search report		Publication date	Patent family member(s)			Publication date
WO 9818931	A	07-05-1998	AU	5194598	A	22-05-1998
			AU	6909098	A	22-05-1998
			EP	0942983	A	22-09-1999
			EP	0941335	A	15-09-1999
			WO	9818930	A	07-05-1998
EP 0885966	A	23-12-1998	CA	2235435	A	20-12-1998
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EP 0891984	A	20-01-1999	CA	2235441	A	20-12-1998
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			AU	6909098	A	22-05-1998
			EP	0942983	A	22-09-1999
			EP	0941335	A	15-09-1999
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			AU	709405	B	26-08-1999
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			NO	960839	A	19-04-1996
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			US	5965141	A	12-10-1999
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			US	5955089	A	21-09-1999
			ZA	9401584	A	12-10-1994

REVISED VERSION*

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(51) International Patent Classification 7 : C12N 15/31, C07K 14/315, 16/12, G01N 33/50, A61K 39/09, C12Q 1/68	A3	(11) International Publication Number: WO 00/06737 (43) International Publication Date: 10 February 2000 (10.02.00)

(21) International Application Number: PCT/GB99/02451

(22) International Filing Date: 27 July 1999 (27.07.99)

(30) Priority Data:

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(71) Applicant *(for all designated States except US)*: MICROBIAL TECHNICS LIMITED [GB/GB]; 20 Trumpington Street, Cambridge CB2 1QA (GB).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: GILBERT, Christophe, François, Guy [FR/GB]; University of Cambridge, Dept. of Pathology, Tennis Court Road, Cambridge CB1 1PQ (GB). HANSBRO, Philip, Michael [GB/GB]; University of Cambridge, Dept. of Pathology, Tennis Court Road, Cambridge CB2 1QP (GB).

(74) Agents: CHAPMAN, Paul, William et al.; Kilburn & Strode, 20 Red Lion Street, London WC1R 4PJ (GB).

(81) Designated States: CN, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published

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(88) Date of publication of the he international search report: 29 June 2000 (29.06.00)

(88) Date of publication of the revised version of the international search report: 9 November 2000 (09.11.00)

(54) Title: STREPTOCOCCUS PNEUMONIAE PROTEINS AND NUCLEIC ACID MOLECULES

(57) Abstract

Novel protein antigens from *Streptococcus pneumoniae* are disclosed, together with nucleic acid sequences encoding them. Their use in vaccines and in screening methods is also described.

*(Referred to in PCT Gazette No. 45/2000, Section II)

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AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

REVISED VERSION

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 99/02451

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/31 C07K14/315 C07K16/12 G01N33/50 A61K39/09
C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K G01N A61K C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 18931 A (DOUGHERTY BRIAN A ;HUMAN GENOME SCIENCES INC (US); ROSEN CRAIG A () 7 May 1998 (1998-05-07) SEQ ID NO 3,5,21,69,127,139,187 ---	1,3-7, 9-19
T	LANGE ROLAND ET AL: "Domain organization and molecular characterization of 13 two-component systems identified by genome sequencing of Streptococcus pneumoniae." GENE (AMSTERDAM) SEPT. 3, 1999, vol. 237, no. 1, pages 223-234, XP004183515 ISSN: 0378-1119 page 229; figures 1,3 ---	1,3-7

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 August 2000	31.08.00

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	ESPEN, J

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GUENZI ERIC ET AL: "A two-component signal-transducing system is involved in competence and penicillin susceptibility in laboratory mutants of Streptococcus pneumoniae." MOLECULAR MICROBIOLOGY 1994, vol. 12, no. 3, 1994, pages 505-515, XP000905352 ISSN: 0950-382X	
P,X	EP 0 885 966 A (SMITHKLINE BEECHAM PLC ;SMITHKLINE BEECHAM CORP (US)) 23 December 1998 (1998-12-23) SEQ ID NO 1,2,3	1,3-7, 9-19
P,X	EP 0 891 984 A (SMITHKLINE BEECHAM PLC ;SMITHKLINE BEECHAM CORP (US)) 20 January 1999 (1999-01-20) SEQ ID No 1,3,4	1,3-7, 9-19
X	WO 98 18930 A (HUMAN GENOME SCIENCES INC ;CHOI GIL H (US); HROMOCKYJ ALEX (US); J) 7 May 1998 (1998-05-07) SEQ ID NO 7,8,113,114,133,134,163,164,169,170	1,3-7, 9-19
P,X	EP 0 887 413 A (SMITHKLINE BEECHAM PLC ;SMITHKLINE BEECHAM CORP (US)) 30 December 1998 (1998-12-30) SEQ ID NOs 1,2,3,4 page 4-6	1,3-7, 9-19
P,X	FONTAN PA ET AL: "Streptococcus pmeumoniae choline transporter" EMBL DATABASE ENTRY AF162656, ACCESSION NUMBER AF162656,26 July 1999 (1999-07-26), XP002136498 nucleotide sequence and deduced amino acid sequence	1,3-7
X	FONTAN P A ET AL: "A choline transporter as a virulence determinant of Streptococcus pneumoniae." 97TH GENERAL MEETING OF THE AMERICAN SOCIETY FOR MICROBIOLOGY;MIAMI BEACH, FLORIDA, USA; MAY 4-8, 1997, vol. 97, 1997, page 103 XP000892162 Abstracts of the General Meeting of the American Society for Microbiology 1997 ISSN: 1060-2011 abstract	1,3-7

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5

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	TAKEMOTO K ET AL: "Putative ferric transport ATP-binding protein AFUC" SWISSPROT DATABASE ENTRY AFUC_ECOLI, ACCESSION NUMBER P37009, 1 June 1994 (1994-06-01), XP002136499 sequence	6,9
Y	FLEISCHMANN RD ET AL: "Putative ferric transport ATP-binding protein AFUC" SWISSPROT DATABASE ENTRY AFUC_HAEIN, ACCESSION NUMBER P44531, 1 November 1995 (1995-11-01), XP002136500 sequence	6,9
Y	BLATTNER FR ET AL: "Spermidine/putrescine transport ATP-binding protein POTA" SWISSPROT DATABASE ENTRY POTA_ECOLI, ACCESSION NUMBER P23858, 1 November 1991 (1991-11-01), XP002136501 sequence	6,9
P,A	POLISSI ALESSANDRA ET AL: "Large-scale identification of virulence genes from Streptococcus pneumoniae." INFECTION AND IMMUNITY DEC., 1998, vol. 66, no. 12, December 1998 (1998-12), pages 5620-5629, XP002136502 ISSN: 0019-9567	
A	DINTILHAC A ET AL: "The adc locus, which affects competence for genetic transformation in Streptococcus pneumoniae, encodes an ABC transporter with a putative lipoprotein homologous to a family of streptococcal adhesins." RESEARCH IN MICROBIOLOGY 1997, vol. 148, no. 2, 1997, pages 119-131, XP002115703 ISSN: 0923-2508	
A	WO 95 06732 A (MASURE H ROBERT ;TUOMANEN ELAINE (US); PEARCE BARBARA J (US); UNIV) 9 March 1995 (1995-03-09)	
A	EP 0 622 081 A (UAB RESEARCH FOUNDATION) 2 November 1994 (1994-11-02)	

...ernational application No.
PCT/GB 99/02451

INTERNATIONAL SEARCH REPORT

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [ ] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. [X] Claims Nos.: 20
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

see FURTHER INFORMATION sheet PCT/ISA/210

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [X] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

1,3-7,9-19 (SEQ ID NO: 1,208; 27,235; 80,292; 132,344; 138,350; 162,178; 166,182)

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[X] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 20

Claim 20 relates to the use of an agent capable of antagonising, inhibiting or otherwise interfering with the function or expression of a protein or polypeptide as defined in Tables 2-4 in the manufacture of a medicament. Neither a true technical characterization is given for such an agent, nor is such an agent defined in the application. In consequence, the scope of said claim is ambiguous and vague, and its subject-matter is not suffieciently disclosed and supported (Art. 5 and 6 PCT).
No search can be carried out for such purely speculative claims whose wording is, in fact, a mere recitation of the result to be achieved.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: in part: 1,3-7,9-19; all as far as applicable

Streptococcus (S.) pneumoniae protein or polypeptide having a sequence relating to SEQ ID No 1, antigenic and/or immunogenic fragment thereof; nucleic acid molecule relating to SEQ ID No 208, and vaccine comprising said nucleic acid; use of said protein or polypeptide as an immunogen and/or antigen; immunogenic and/or antigenic composition comprising said protein or polypeptide, and its use as a vaccine; antibody directed to said protein or polypeptide; method for the detection/diagnosis using either said protein/polypeptide, or said antibody, or said nucleic acid molecule; method of determining whether said protein or polypeptide represents a potential anti-microbial target

2-179. Claims: in part: 1-19; all as far as applicable

as invention 1 but limited to subject-matter relating SEQ ID Nos 2-151 (table 2), SEQ ID Nos 152-167 (table 3), and SEQ ID Nos 184-195 (table 4) and the corresponding nucleic acid molecules; wherein
invention 2 is limited to SEQ ID No 2,
invention 3 is limited to SEQ ID No 3, etc...
invention 179 is limited to SEQ ID No 195.

International Application No
PCT/GB 99/02451

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